Physiological Zoölogy

Vol. XII OCTOBER 1939 *No. 4*

GROWTH STUDIES ON CILIATES. I. THE ROLE OF BACTERIA IN THE GROWTH AND REPRODUCTION OF COLPODA[1]

(Two figures)

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THE various species of the genus *Colpoda* have been the subject of numerous and interesting investigations for many years. From the standpoint of the cell physiologist these ciliates offer unusual material in that they are relatively easy to culture, they have a high-division-rate, and they form permanent cysts. This last fact has made them the subject of a great many investigations regarding the nature and causes of encystment and excystment. Of the various attempts to analyze these problems none, we feel, have been done under conditions which would permit a proper control over extraneous factors, of which a principal one is associated bacteria. We have succeeded in controlling this factor and along with it many others that enter into the problem.

It is the purpose of this report, which is the first of a series, to detail our experiments which have been carried on for well over a year. It is hoped that we can show the necessity of, and the consequences of the lack of, a precise method for bacterial control in dealing with the culture methods for studies of ciliate metabolism in general. While the majority of the data given regarding the effects of differential feeding are of a qualitative nature, the observations here recorded are, we believe, of extreme significance.

Our strain of the ciliate *Colpoda*[2] came from a single individual isolated from an infusion of Florida hay in the same manner as previously described for *Colpoda cucullus* (Kidder and Claff, 1938). The progeny of this individual were grown for several months on boiled hay infusion, and careful records were made of the growth-rate, time of encystment, time and method of excystment, as well as of the cytological details during the

[1] This investigation was supported in part by a grant-in-aid from the Society of the Sigma Xi.

[2] Since the completion of this study we have received cultures of *Colpoda duodenaria* from Dr. C. V. Taylor, and it appears that our strain is morphologically identical with it. An analysis of the genus is now in progress in this laboratory, and until this is completed we should prefer to withhold specific designation of our strain.

various phases of the life-history. These records have been used for comparative purposes throughout the later periods of the investigation.

In January, 1938, the initial investigations into the effects of bacteria on the growth, reproduction, and cyst formation began. Because of previous experience in the feeding of bacteria to *Moina macrocopa* (Stuart *et al.*, 1931) it was decided that a likely food organism with which to start would be *Aerobacter cloacae*, a common coliform bacterium found abundantly in soil and water. This bacterium has proved to be not only adequate as food for our strain of *Colpoda* but far superior to any other type or combination of types which we have used.

It is obvious and has been mentioned many times in the literature within the past few years that in order to test the effects of any given food material on a bacteria-feeding protozoan, that protozoan must be absolutely freed of contaminating microörganisms. Our first attempt at sterilization consisted of a method of washing single trophic ciliates in ten changes of sterile medium, either dilute proteose-peptone broth, hay tea, or distilled water, transferring between each bath with sterile micropipettes. All dishes were sterilized before use and kept covered throughout the process except for the time needed to effect the transfer. This method is similar to that described by Hargitt and Fray (1917) and Parpart (1928). After the tenth transfer, the whole process consuming about 3 hours, the individual ciliates were placed in bacteriological tubes containing different media.

We were anxious to prevent, as far as possible, the growth of any contaminating microorganisms as well as those bacteria which we were presenting as food. To this end we set up tubes containing *Aerobacter* suspended in Pyrex distilled water, without the addition of any of the salts which had previously been used in studies of this kind (see Barker and Taylor, 1931). We wanted to test the theory that, even if our method of sterilization proved to be ineffective, it would, nevertheless, reduce the number of contaminating microörganisms to such a low number that they would be eliminated in successive transplants in *Aerobacter* suspensions. This method of substitution of bacterial types had been previously reported by Oehler (1920).

It was found that all the ciliates, washed as previously described and placed in nutritive media, showed macroscopic contaminations within 48–72 hours. Therefore, the method used was ineffective for complete sterility. Those ciliates placed in suspensions of *Aerobacter* in distilled water grew exceedingly rapidly and to enormous concentrations. At the end of 48 hours the fluid had become crystal clear, most of the *Aerobacter* having been eaten, and most of the ciliates had encysted in a ring about the tube at the surface. Here, then, was a situation which should yield results in the elimination of contaminants by transplantation. Accordingly, serial transplants were made into fresh *Aerobacter* suspensions by means of a bacteriological loop. A number of series were started in this manner, transplants being made every 48 hours. At the outset agar plates were streaked from these tubes, and contaminants were found but only in small numbers. These numbers indicated that if the fluid was actually non-nutritive these organisms would be speedily lost when transplanted through suspensions of billions of *Aerobacter*.

After a period of 6 weeks, stock was taken of our results. It was seen at a glance that something was changing in our serial transplants. Whereas the early tubes had given remarkable ciliate growth and the fluid had become clear, as time went on the ciliate growth decreased in direct proportion to the failure of the fluid to become clear after encystment had taken place. What was the cause of the persistence of cloudiness in the

later tubes? Were the ciliates failing to eat the *Aerobacter?* Agar plates streaked from these tubes demonstrated a point of great importance. These plates showed very few *Aerobacter* but were covered with extraneous types—the types which were brought in as contaminants in small numbers with the original washed ciliate. These contaminants, then, had actually increased in number to an enormous degree while the ciliates were ingesting *Aerobacter*. Upon what were the contaminants feeding, and why were they not eaten out at the same rate as were the *Aerobacter?* It seemed obvious that the *Colpoda* must be practicing some kind of selectivity in their feeding and that the contaminants were growing, possibly on the metabolites of the ciliates or on the disintegration products of the bacteria or both. Certain it was that the fluid had become nutritive during the period of ciliate growth. This meant, therefore, that at each transplant the contaminant inoculation would be, even in a loop, heavier and heavier. The conclusions suggested by this observation—namely, that the fluid was nutritive after the ciliates had grown in it, that the ciliates selected *Aerobacter* as food in preference to the contaminants in these tubes, and that the contaminants appeared to be detrimental to the growth and well-being of the ciliates—were subjected to experimental check.

TABLE 1

EXPERIMENT TO TEST THE ABILITY OF TWO TYPES OF FLUID TO SUPPORT GROWTH OF *Pseudomonas*

Fluid	Bacterial Counts				
	Initial	24 Hours	48 Hours	72 Hours	96 Hours
1. Filtered ciliate culture*	45,000	4,500,000	29,600,000	23,900,000	30,000,000
2. Filtered *Aerobacter* suspension†	25,000	38,000	165,000	1,480,000	21,500,000

* Filtered (Seitz) *Colpoda-Aerobacter* culture 48 hours after growth of the ciliates had started.
† Filtered (Seitz) *Aerobacter* suspension which had stood 48 hours previous to filtering.

In order to demonstrate the nutritive quality of the fluid in which ciliates have grown, the following experiments were performed. Forty-eight-hour cultures from our *Colpoda-Aerobacter* pure series (to be described later) were drawn through a Seitz bacteriological filter, and the filtrate inoculated with *Pseudomonas* (our most prevalent contaminant). The initial inoculum was determined by plate count, and the tubes incubated at 26° C. Thereafter bacterial counts were made at 24-, 48-, 72-, and 96-hour intervals. A parallel series using a filtrate of distilled water in which *Aerobacter* alone had been suspended for 48 hours before filtration was inoculated and treated in the same manner as the first. The results of these experiments are presented in Table 1 and are graphically represented in Figure 1.

The marked increase of the bacteria in the *Colpoda-Aerobacter* filtrate might conceivably be due to nutritives derived from the ciliate metabolites, bacterial disintegration products, traces of medium brought in from the agar slants during the initial bacterization 48 hours previous to filtration, or to a combination of these factors. Ciliate metabolites are eliminated from the second set of tubes, and the growth here is much slower and the concentrations lower during the first 72 hours. By the end of 96 hours the concentrations are nearly equal. It must be remembered, however, that the presence of

Aerobacter in high concentrations for a period of 48 hours (as was the case in the second part of this experiment) never occurs in the ciliate cultures, as they are rapidly eaten out. Therefore, it is not correct to compare this experimental fluid to that of a ciliate culture. Judging solely from the figures, however, it appears that a considerable quantity of nutrient material, over and above that supplied by bacterial disintegration, is supplied by some product of ciliate activity, together with what nutrients may have been brought in with the original bacterization. We believe that the nutrients brought in from the agar slants are negligible, considering the fact that the bacteria are always removed with care with a wet loop. Distilled water inoculated with *Pseudomonas* showed a slight decrease in numbers at the above-mentioned time intervals.

We feel that the foregoing results are quantitatively conservative as compared to our first observations on the growth of the contaminants in our tubes, because it must be remembered that we were testing the nutrient qualities of the fluids with bacteria previously grown on agar slants. These inoculated bacteria were probably not as well adjusted to the experimental fluids as were those which had grown for 6 weeks in association with the ciliates.

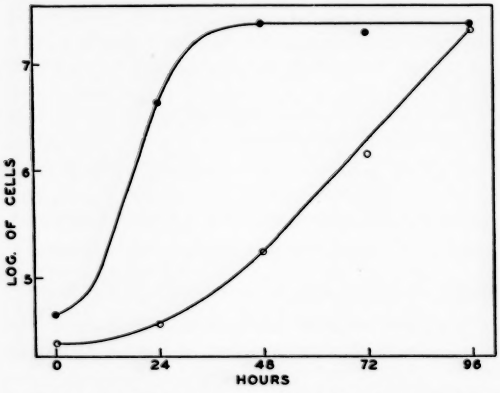

FIG. 1.—Graphic representation of the data contained in Table 1.

Testing the other points suggested by our observations necessitated complete sterilization of the ciliates. This was effected by centrifugation and dilution. It was found, however, that slight but important modifications had to be made in the usual method in order to obtain the desired results. Our procedure, as finally perfected, consisted of growing large numbers of ciliates in tubes of distilled water bacterized with *Aerobacter*. When the ciliates had encysted, these tubes were emptied of their fluid, and the cysts, adhering to the walls, were rinsed in many successive changes of sterile distilled water using aseptic technique. This manipulation aided in removing gross contaminations. These tubes were then filled with sterile distilled water to a point where the cyst rings were just submerged. They were allowed to stand until the ciliates had excysted, and the small trophic forms were swarming in the water. Under these conditions excystment took place in about an hour, although there were some irregularities in time, the causes for which are under investigation at the present time. The advantages of this procedure were that an opportunity was offered to pre-wash the cysts and that the cytoplasm of the freshly excysted ciliate was devoid of food.

After excystment had occurred, the ciliates were concentrated by slow centrifugation, and the concentrate removed to a single, sterile, cotton-plugged centrifuge tube. This ciliate concentrate was diluted with 10 ml. of sterile distilled water and recentrifuged at a speed which would just throw down the majority of the ciliates in 3 minutes. It was found that the most satisfactory speed for this purpose was 2,000 revolutions per minute.

As soon as the centrifuge stopped, the supernatant fluid (9 ml.) was immediately withdrawn with a sterile 10 ml. pipette, and the tube was allowed to stand for about 2 minutes in order that the ciliates might swim to the top of the remaining milliliter of water. With a sterile 1 ml. pipette, 0.5 ml. of ciliate suspension was withdrawn and placed in an empty sterile centrifuge tube. This suspension was again diluted, and the process repeated until the ciliates had gone through an average of fifteen such transfers with the accompanying dilutions (a dilution factor of approximately 10^{14}). This method entails a great loss of ciliates but was found necessary inasmuch as, without removal to fresh tubes with the consequent discarding of the residue, contamination was invariable. It was demonstrated that the contaminations resulted from the fact that ciliates died or became immobilized during centrifugation and were carried passively to the bottom of the tube with their adhering bacteria. However, by discarding the dead forms we were able to sterilize several hundred ciliates at each attempt, and these gave us the necessary organisms with which to work.

The details of our methods have been given here in the hope that they may prove useful to future investigators. In our opinion it is a fallacy to assume that the removal of bacteria from any free-living protozoan can be accomplished by any one standardized method. Each type will have to be treated individually and its peculiarities studied in order to determine a satisfactory method. And in order to be sure of the end results each step in the manipulation should be checked with bacteriological broth inoculations and poured or flooded (Anderson and Stuart, 1935) agar plates, these being kept for a *minimum of 14 days*. It is only by employing these rigid checks that the slow-growing bacterial contaminants can be detected. All the foregoing precautions and checks were adhered to in our work.

SERIAL TRANSPLANTS IN *Aerobacter* SUSPENSIONS

Starting with sterile ciliates, three series of bacteriological tubes were set up in which were placed 6 ml. of distilled water bacterized with *Aerobacter*. These tubes behaved as did the previous early tubes of the contaminated series, enormous growth of the ciliates with a consequent cleaning-out of the *Aerobacter* taking place within a period of 48 hours, the ciliates then encysting on the walls of the tubes. Just as encystment began, one bacteriological loop of this ciliate culture was transferred aseptically to a fresh tube of each series. Unlike the contaminated series, however, no diminution of ciliate growth took place in the following weeks, and the ciliates did not fail to clear out the fluid. To date these series have been running for over a year through 192 transplants, without showing any decrease in numbers per tube at the end of the 48-hour periods, except in a few instances where the stock *Aerobacter* culture dissociated from the normal smooth form to either rough or mucoid variants. Separate experiments show that neither of these variants of *Aerobacter* is as satisfactory as the normal form.

STERILE CILIATES IN PURE CULTURES OF CHROMOGENIC BACTERIA

Single sterile ciliates were transferred into distilled-water, hanging-drop suspensions of the various chromogenic bacteria which had been isolated from the contaminated tube series, in order to test their specific effects upon growth and reproduction. The hanging-drop method was employed that they might be observed directly.

Along with these experimental slides, controls on the sterility of the ciliates were set up as follows: four or more single ciliates were placed in hanging drops of proteose-

peptone broth and four or more in distilled water. After every tenth experimental setup, broth cultures were again prepared, and this was repeated at the end of the total number. In this way we could check microscopically, in a relatively short period of time, any gross errors in our procedure, as well as the initial sterility of the ciliates. After the experiments were finished, the final check of our drops came from streaking out on plates and examining for possible contaminants. By working in a room devoid of air currents,

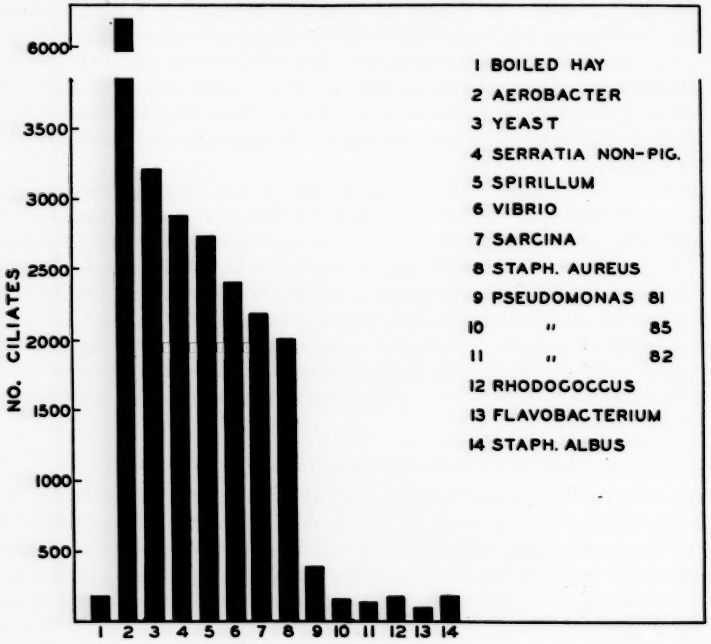

FIG. 2.—Graphic comparison of the total number of cells produced at the end of 48 hours in hanging-drop suspensions of various food organisms. The suspensions were of approximately equal densities (about 10 billion per cc.) at the start of the growth periods. The initial number of *Colpoda* in each case was 1, and the volume of medium was approximately 0.005 ml. All ciliates were completely sterile at the start of the experiments, except No. 1 (boiled hay).

by using only sterile equipment, by performing all manipulations under a Cellophane hood, and by making all transfers under a dust shield covering the stage of the dissecting microscope, we were able to eliminate chance contaminations to less than 1 per cent of the total number of experiments. Those which were found to contain contaminants were so recorded, and the results obtained were discarded.

The chromogens tested can be grouped into three categories in regard to their effects on the ciliates: those which were eaten by the ciliates but produced poor to mediocre growth (as compared to *Aerobacter*); those which were entirely rejected as food but which produced no abnormalities; and those which proved to be toxic.

In the first category the following types were found: *Pseudomonas* (three species designated by us as Nos. 81, 82, and 85), *Staphylococcus aureus*, *S. albus*, *Spirillum rubrum*, *Sarcina lutea*, *Rhodococcus roseus*, and *Flavobacterium* (two species, one orange and one lemon yellow). While the ciliates fed upon these bacteria, in no case did the growth equal one-half that attained when *Aerobacter* was presented as food in comparable concentrations. The relative abilities of these chromogens to support the growth of *Colpoda* can be seen from Figure 2. This figure is, however, misleading as to the end result of the cultures. The numbers given are the number of cells produced at the end of 48 hours, but the culmination of a healthy culture is the formation of resistant cysts. In the case of cultures of *S. albus* and *R. roseus* not only were abnormalities observed in the trophic organisms but all the ciliates died without forming cysts. While cysts were produced in cultures of the three species of *Pseudomonas*, excessive numbers of abnormalities occurred in the form of monsters, a great many of which perished without encysting. In the remaining types, although the growth was limited, encystment always resulted in the normal manner.

In the second category we found only one type, *Flavobacterium* sp. (our orange No. 3). Sterile ciliates placed in suspensions of this bacterium behaved precisely as did those placed in sterile distilled water. In most cases encystment took place after about 36

TABLE 2

BACTERIA NOT SUPPORTING GROWTH

Flavobacterium (orange No. 3)	Nontoxic
Chromobacterium violaceum	Toxic (trophic and cyst)
Serratia marcescens (pigmented)	Toxic (trophic and 90 per cent cysts)
Aerobacter cloacae (autoclaved)	Nontoxic
A. cloacae (killed 100° C.)	Nontoxic
A. cloacae (killed 55° C.)	Nontoxic
Serratia marcescens (autoclaved)	Toxic

hours, the single ciliate having become appreciably smaller. Spontaneous excystment occurred, and the single ciliate, after a period of swimming, would again encyst. Each cyst was smaller than the previous one until, at the end of 6–8 days, the tiny, rounded form would disintegrate. From a careful examination of many ciliates under the highest powers of the microscope we are of the opinion that none of this species of bacteria were ingested by the ciliates. Whatever may be the cause of the rejection of this bacterium, it does offer an explanation for the persistence of this type of *Flavobacterium* in increasing numbers in our contaminated series.

The third category of bacteria proved to be the most interesting from the standpoint of the cultural restrictions. These experiments are presented in Table 2. Sterile ciliates placed in suspensions of pigmented *Chromobacterium violaceum* or *Serratia marcescens* were invariably killed in 2–30 minutes, depending upon the concentration of the bacteria. Both these types are known to produce what has been called a nondiffusible pigment, insoluble in water but soluble in alcohol, chloroform, or ether. It was at first assumed that the toxic effects were produced as a consequence of ingestion of the bacteria by the ciliates, but it was later demonstrated that this could not be the sole explanation. Because of the different effects produced by the two types of bacteria, they will be discussed separately.

In suspensions of *C. violaceum*, taken from an agar slant where the pigment was pronounced, all trophic *Colpoda* exhibited a marked change in their swimming activities.

They became flattened and twisted and moved more and more slowly. Very shortly a distinct lavender flush could be seen throughout the cytoplasm, and motion ceased. Within the space of a few minutes after they became motionless disintegration occurred. This was so striking that large numbers of ciliates from *Aerobacter* tubes were introduced into drop suspensions. The same results were obtained and in about the same time. No protective effect was apparent either from the *Aerobacter* present or from the large numbers of ciliates. Next, large numbers of resistant cysts were placed in drop suspensions of *C. violaceum*. Approximately 99 per cent of these cysts became lavender in color, the color concentrating in the cytoplasm and leaving the cyst wall clear. These cysts showed no protoplasmic activity, and, although they did not disintegrate, they never excysted. The remaining 1 per cent went through a slow but rather normal excystment stage, and the emerging trophic forms were immediately killed.

From the results obtained on the resistant cysts it seems quite clear to us that the toxicity was in some way bound up with the purple pigment of the bacteria. It was also evident that, at least in association with the ciliates, the pigment could and did diffuse out of the bacterial cell, otherwise it could not have made its appearance within the cyst wall. This point was well illustrated when the experiments were repeated, using *Serratia* suspensions.

In suspensions of pigmented *Serratia* trophic ciliates were always killed, as in the previous experiments. The toxicity was manifested in a slightly different way, however, in that the ciliates became rounded but retained their powers of motility. The cytoplasm became more and more pink, the contractile vacuoles became greatly distended, and the ciliates burst very suddenly. When cysts were placed in suspensions of *Serratia* about 90 per cent of them reacted in the same manner as those in suspensions of *Chromobacterium*, except that the cytoplasm became pink at first, then changing to a deep red as if the pigment was being concentrated from the suspension. Here again it appeared that the pigment was diffusing from the bacterial cells and entering the cyst walls. The other 10 per cent remained colorless, excysted in a perfectly normal manner, and upon emergence as trophic ciliates were "immune" to the pigment. This 10 per cent had, in some way, become acclimatized to the pigment and were able to feed and grow upon the *Serratia*. Nor was this acclimatization a matter of cell selection, for very often in double resistant cysts, one sister-cell would become blood red and die while the other sister-cell would remain colorless and would excyst. From these acclimatized ciliates it was possible to start subcultures in heavy suspensions of the bacteria.

It should be noted that the pigment of *Serratia* varies to a considerable degree under different cultural conditions. We have found that *Serratia* grown on nutrient agar produces an orange-red pigment, while those grown on nutrient agar to which 1 per cent glycerine has been added always produce a pink pigment at first, turning to a deep, purplish red. While both of these pigments are toxic, distinct differences in their effects on the ciliates have been noted.

An indication of the answer to the question of pigment as the active toxin was found when sterile trophic ciliates were placed in suspensions of nonpigmented *Serratia*. These were entirely unharmed and were able to feed on these bacteria in a normal manner (see Fig. 2).

That the toxic effect of the pigment is not dependent upon the living *Serratia* and that its toxicity is not destroyed by heat is demonstrated by the fact that autoclaved suspensions of the bacteria proved to be fully as toxic to *Colpoda* as were the live bacteria in suspension.

Recently we have obtained some evidence pointing to a partial explanation of these puzzling phenomena in so far as they relate to the pigment of *Serratia*. It was found that resistant cysts that had been washed in 50–100 ml. of distilled water and then placed in suspensions of pigmented *Serratia* were not killed, but all excysted and then were able to feed unharmed on the bacteria. This would suggest that something is liberated from the trophic ciliate into the medium, capable of acting upon the bacterial cell causing it to liberate its pigment in greater quantity than normal. This high concentration is lethal to all trophics and to 90 per cent of the cysts. This product of the ciliate growth in the medium previous to encystment is presumed to adhere in considerable concentration to the cyst wall. Being soluble, it is removed from the cyst by washing, and the concentration of the pigment outside the bacterial cells remains low during the process of excystment. Upon excystment the ciliates have become acclimatized to the sublethal concentration of pigment normally present in the suspension and, although they presumably secrete more of the substance, are from then on unharmed. The presence of the substance is further demonstrated by placing washed cysts in suspensions of *Serratia* made by using the supernatant fluid from a growing *Colpoda* suspension instead of distilled water. Here the majority of the cysts are killed, as in the case of the unwashed cysts, indicating that enough of the substance is present in the fluid of the growing culture to liberate a lethal quantity of pigment.

It is instructive and interesting to note that similar results were obtained with the nonencysting *Colpidium campylum*. This ciliate, grown bacteria free in a proteose-peptone broth or on the surface of proteose-peptone agar slants, exhibits varying degrees of sensitivity to the toxic action of the pigment of *Serratia*. If a drop of broth is bacterized with pigmented *Serratia* and a few *Colpidium* from a broth culture are introduced, the ciliates are quickly killed, first becoming pink and round. Such is not the case, however, if the *Colpidium* have been removed from an agar slant. They are able to acclimatize themselves to the pigment and grow and reproduce in a normal manner. Now if a broth culture of *Colpidium* is centrifuged, the supernatant fluid used for the *Serratia* bacterization, and agar-grown ciliates introduced into this type of drop, they are all killed.

Here, again, it appears that the ciliates are releasing a substance which may act upon the *Serratia*, causing them to release an abnormally high concentration of pigment. The substance seems to be contained in the broth in which the ciliates have grown. When the ciliates are grown on agar slants, this substance possibly diffuses into the agar and, therefore, would be nearly absent from a loop of these ciliates. But the agar-grown ciliates are nevertheless killed if the substance is provided from a broth-culture centrifugate.

In the case of *Chromobacterium violaceum* suspensions, like experiments yielded similar results but with this difference. Washed cysts of *Colpoda* were found to excyst about 100 per cent, but there followed no acclimatization of the trophic ciliates. All trophic ciliates were immediately killed upon emergence.

THE PIGMENT OF *Pseudomonas*

To revert momentarily to the results obtained with suspensions of *Pseudomonas*, it will be recalled that *Colpoda* grew poorly when feeding on any one of the three species used. What is the relation between the pigment of these types and the pigments of *C. violaceum* and *S. marcescens* in regard to toxicity? We know that the pigment of *Pseudomonas* is diffusible and that bacteria removed from an agar slant leave their pigment behind. Therefore, the ciliates in our experiments would be coming in contact with only a minute quantity of the pigment. To test the activity of concentrated pigment, broth

tubes were inoculated with the three species of *Pseudomonas* used. Within 72 hours a well-defined ring of the blue-green pigment had collected at the top of the tubes. From some of this pigment hanging drops were prepared, and trophic ciliates added to them. They were instantly killed. When cysts were used they, too, were killed. It was found that even in great dilutions the pigments of all three species of *Pseudomonas* were lethal, the length of time before death varying with the concentration. No attempt has been made as yet to determine which fraction of this well-known pigment is responsible for the death of the ciliates. The point of importance for our present consideration is the fact that growth products of such a common soil-and-water form as *Pseudomonas* can produce such a drastic effect upon the ciliates. This observation corresponds in part with that of Cleveland (1928) working with *Tritrichomonas fecalis*. He reported the extreme toxic effects on this protozoan of "exotoxins" of *P. fluorescens* and *P. aeruginosa*.

DEAD BACTERIA AS A SOURCE OF FOOD

In the course of our work we have had occasion to test the possibility of feeding *Colpoda* on dead bacteria. We prepared suspensions of heat-killed *Aerobacter*, killed in three different ways: at 55° C. for 1 hour, at 100° C. for 30 minutes, and autoclaved for 15 minutes. Into hanging drops of these suspensions were placed sterile ciliates. In two cases where *Aerobacter* killed at 55° were used, fair ciliate growth resulted. Both of these drops when plated showed live *Aerobacter*. All other experiments failed to show any ciliate growth and proved to contain no living bacteria when plated. The ciliates behaved in the same manner as those placed in the distilled-water controls. These experiments were repeated on autoclaved, nonpigmented *Serratia* with the same results. In short we have never observed any growth whatsoever in our strain of *Colpoda* in the absence of living food, either in the above-mentioned suspensions of dead bacteria, broths of various types and concentrations, or yeast extracts. The possibility of finding the exact combination of nonliving substances and conditions which will satisfy the nutritional requirements of this ciliate is not ruled out, however, and work is progressing with that object in view.

DISCUSSION

It is unnecessary here to review the work on the sterilization of various species of protozoa inasmuch as this has already been done a number of times before (see Phelps, 1934; Johnson, 1936; Hall, 1937). Space will be devoted, therefore, only to a discussion of the problems relative to the experimental work reported here.

We consider our findings on the ability of extraneous bacteria (many of which are detrimental to ciliate growth) to maintain themselves and increase in numbers in serial transplants to be of significance in any cultural work. We believe that this demonstration alone points out a possible cause of "depressions" in cultures under uncontrolled conditions and may point the way to a revaluation of some of the life-cycle work of the past. It indicates that it is entirely erroneous to assume, because only a few extraneous bacteria are introduced with an organism into a culture medium, although that culture medium may consist of a suspension of a food organism in a non-nutritive fluid, that these few will produce no effect. It is not only probable but inevitable that some form of food selectivity will be exhibited by the experimental animals and that there is always the probability of harmful forms persisting and increasing to a proportion where their effects will be felt. We cannot, therefore, subscribe to any notion of "relative sterility" in preparing protozoa or any other type of bacteria-feeder for experimental use.

It has been pointed out by Chatton and Chatton (1927*a*; 1927*b*) that certain chromogenic bacteria (*B. fluorescens* and *B. prodigiosus*, which are our *Pseudomonas* and *Serratia*) exert a cytolytic effect upon *Glaucoma scintillans*, *Colpidium colpoda*, *Colpoda steini*, *C. cucullus*, and *Paramecium caudatum*. They noted that this effect was most pronounced in highly pigmented strains but thought that the toxicity was not due to the pigment itself but was a combination of a glucoside and a lipoid. Their experimental evidence upon the nature of the toxic substance is incomplete. It should be noted in this connection that Phelps (1934) was able to grow *P. aurelia* on pure strains of pigmented *Serratia* (*Erythrobacillus prodigiosus*) without noting any harmful effects. There appear to be two possible explanations for these results: (1) that he was able to effect acclimatization in his strain of protozoa to the *Serratia* pigment or (2) that that species of protozoa was not reactive to the pigment. The latter explanation seems the less probable in view of the results obtained by the Chattons on another species of the same genus.

Taylor and his co-workers (Barker and Taylor, 1931, 1933; Thimann and Barker, 1934; Taylor and Strickland, 1935, 1936, 1938; Taylor, Brown, and Strickland, 1936) have contributed a number of interesting reports concerning the growth, encystment, and excystment of *Colpoda*. The species of *Colpoda* used in all these studies should be designated as *C. duodenaria* according to Taylor and Furgason (1938), although it was earlier described as *C. cucullus* and *C. steini*. In these investigations the ciliates were fed on a suspension of *Pseudomonas* in a balanced salt solution with the idea that the bacteria would not increase in numbers during the experiments and that any extraneous bacteria would have no effect because they also would fail to multiply. We have already pointed out that these assumptions are not necessarily correct.

Relative to the question of the ability of *Colpoda* to feed and reproduce by using only dead bacteria as a source of food, we can only harmonize the findings of Oehler (1919, 1920) with our own by the conviction that his ciliates were never entirely free of bacteria when tested. In our experimental setups with dead bacteria we never obtained any growth. Growth resulted in those cultures where, owing to the treatment of the *Aerobacter* (heating at 55° C.), not all the bacteria were killed. Those few grew and were in turn eaten by the ciliates, producing the appearance of growth by feeding on the dead bacteria. In every case, however, where ciliate growth took place, plating disclosed the presence of viable bacteria. The method employed by Cleveland (1928) of growing *Tritrichomonas fecalis* anaerobically on dead bacteria would not be expected to yield results with *Colpoda* when we consider the relatively high oxygen requirements of this ciliate.

It is interesting to note that the food requirements of this strain of *Colpoda* and the reaction of this protozoan to bacterial pigments parallels to a surprising degree those of the cladoceran *Moina macrocopa* (Stuart *et al.*, 1931) and appears to be quite different from those types of ciliates which have been grown in nutrient, bacteria-free broths. This comparison, along with the possession of a mode of reproduction within a cyst wall, seems to point to the classification of *Colpoda* as a highly specialized member of its group.

For the sake of completeness we have included in our Figure 2 a number of organisms tested in a pure state which have not been included in the general discussion. Obviously we have tested only a small proportion of the types of bacteria which would be commonly met with in samples of hay infusions. To test them all would be an impossible task and would probably add little to the value of the work. We feel that this portion of the work demonstrates our points, which are, in summary:

1. Distilled water becomes nutritive for many bacterial types as it becomes the solvent for ciliate metabolites and bacterial disintegration products.

2. Of these many bacterial types some may prove to be deleterious to the ciliates when they reach appreciable concentrations.

3. Deleterious concentrations of bacteria (certain types) may be reached even in serial transplants because of the selectivity of feeding on the part of the ciliates and the accumulated increase in size of contaminant inoculum from transplant to transplant.

4. Certain bacterial pigments are toxic to *Colpoda*, even in low concentrations.

5. *Colpoda* possesses the ability to become acclimatized to the red pigment of *Serratia marcescens*.

6. This strain of *Colpoda* failed to utilize dead *Aerobacter* as food.

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GROWTH STUDIES ON CILIATES. II. THE FOOD FACTOR IN THE GROWTH, REPRODUCTION, AND ENCYSTMENT OF COLPODA[1]

(Three figures)

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THE experiments to be reported here were designed to determine quantitatively the potentialities of growth of the ciliate *Colpoda* in distilled water bacterized with the coliform organism *Aerobacter*. The *Colpoda* used were taken from our stock strain, described in Part I of this series (Kidder and Stuart, 1939), maintained on a pure culture of *Aerobacter*. All experiments were performed with aseptic technique, and the same care was taken to prevent contaminations as was outlined in our previous work.

Our earlier experiments showed that when *Aerobacter* alone was presented as food the ciliates exhibited an extremely high reproductive rate. Under a given set of conditions what are the limits of growth of these ciliates and what are some of the factors which operate to determine these limits? Many reports in the literature deal with these questions but, aside from the older isolation culture methods, few have considered them from the standpoint of a single protozoan. We have evidence to show that survival and growth may vary depending upon the initial number of protozoa used. Accordingly, all of our experiments have been started with single trophic ciliates, and our results are designed to demonstrate the potentialities of growth exhibited by these isolated cells under varying conditions of food concentration in a given volume and in varying volumes of a given food concentration. We are making no attempt in this report to analyze our results in relation to possible growth-promoting factors similar to the allelocatalytic agents of Robertson (1921, 1927). Our evidence along these lines will be given later in the series.

Colpoda offers excellent material for investigations of this type because of its ability to form resistant cysts. A culture may be started, and the end result of growth may be determined at any time after growth has ceased, by counting the number of cysts formed. This fact has proved to be of great value where the hanging-drop method is employed. In larger volumes, however, it is impossible to count the cysts directly, but by adding fresh fluid excystment can be induced and the trophics then counted. In other words, the cysts form a permanent record of the growth that has taken place and can be used for counting at any time after growth has stopped.

In addition to the hanging-drop technique we have used sterile 10×40 mm. shell vials containing the varying volumes of bacterial suspensions. With a sterile micropipette one ciliate from stock was introduced into the suspension, and the vial corked with a rubber stopper. It was necessary when the volumes were small to prevent evaporation; so to this end sterile wet cotton plugs were inserted below the rubber stopper to within about 2 mm. of the culture. Each experimental series was set up by tens and repeated three times on successive occasions. Cultures were grown at room temperature (22°–24° C.). The method of counting ciliates was as follows: During the growth period sets of vials were select-

[1] This investigation was supported in part by a grant-in-aid from the Society of the Sigma Xi.

ed, appropriate dilutions of the contents prepared, and the ciliates, in samples of known volume, were counted with the aid of a dissecting microscope. At the end of the growth period when the ciliates had encysted, the fluid was removed and 0.5 ml. of distilled water was added. After excystment was complete, known volume samples were counted, as above. All counts were repeated, and averages taken.

0.005 ML. VOLUMES; VARYING FOOD CONCENTRATIONS

A 1 ml. suspension containing approximately 10 billion *Aerobacter* in distilled water was prepared, and from this suspension six graded dilutions up to 1:100,000 were made. These dilutions were numbered 2–7, from the greatest to the 1:1 (10 billion) series respectively (see legend of Fig. 1). Hanging drops (approximately 0.005 ml. in volume) of each of the six suspensions were prepared in duplicate on sterile cover glasses, and a single ciliate added to each drop. At the end of 48 hours all ciliates had increased in numbers, had exhausted the food supply, and had encysted. By using the mechanical stage of the compound microscope and a hand counter, every cyst in all drops was counted, and the average number taken for each dilution. This experiment was repeated on three successive occasions.

It was found that the average number of ciliates produced in the 1:1 (10 billion *Aerobacter* per ml.) suspension was 6,280 and that the averages decreased as the dilution increased (Fig. 1). In all cases the *Aerobacter* had been removed to a point where none could be detected in the fluid, although a few were still adhering to the cover glass and the surface film. In distilled-water controls (dil. 1, Fig. 1) the ciliates divided so that there was an average of 8 at the end of 48 hours. This multiplication in distilled water is explained by the presence of a few *Aerobacter* introduced with the ciliates (taken from *Aerobacter* cultures) and also by the presence of a certain quantity of unassimilated food within their bodies.

In all the foregoing dilutions the ciliates grew in proportion to the amount of available food (Fig. 1). What would be the result of increasing the concentration of bacteria to a point where all could not possibly be eaten? We prepared hanging drops of distilled water bacterized to the consistency of heavy cream and into these drops placed single ciliates. All the ciliates lived and reproduced at a rate even higher than in the 1:1 suspension. At the end of 48 hours, the time at which the ciliates in the 1:1 suspension encysted, over 20,000 ciliates were present in the "heavy cream" (dil. 8, Fig. 1). Not all the *Aerobacter* were removed, however, and the end was not encystment but sudden rupturing. Just before the ciliates ruptured, the number was so great within the confines of the 0.005 ml. drops that the ciliates were touching one another. We repeated these experiments many times and always with the same results. Obviously we had given the ciliates more food than they could use within such narrow spatial limits. It might be asked, if it were merely a matter of space within which to grow, why, then, after a number of ciliates had disintegrated would not this decrease in protoplasmic bulk allow those intact ciliates to live, feeding on the remaining *Aerobacter?* The answer to this question is that the products of ciliate disintegration are extremely toxic to fresh ciliates and also produce very rapid bacterial agglutination. Into many of these drops were placed from one to hundreds of additional fresh ciliates. These always ruptured within a few minutes.

0.5 ML. VOLUMES; VARYING FOOD CONCENTRATIONS

It appeared from the results of the foregoing experiments that we might gain some valuable information regarding reproductive potentialities if we increased the volume

of the suspensions, thereby increasing the number of bacteria available in lower concentrations. To that end we prepared seven series of vials, each series being a dilution of a

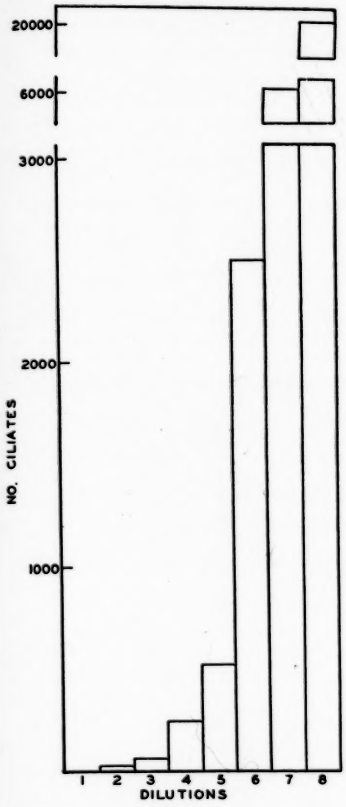

FIG. 1

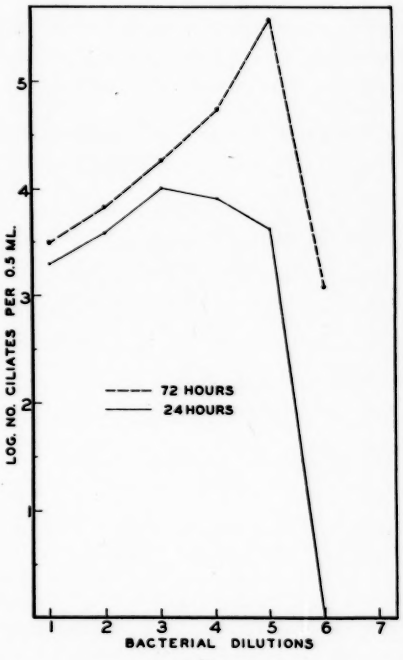

FIG. 2

FIG. 1.—Graphic representation of data obtained from the growth of single ciliates in 0.005 ml. volumes of varying concentrations of *Aerobacter* in distilled water. Direct counts of ciliates made at the end of 48 hours. No. 1 is distilled-water control; No. 7 is a suspension of 10,000,000,000 *Aerobacter* per ml.; Nos. 2–6 are dilutions of No. 7 by tens (No. 2 = 100,000, No. 3 = 1,000,000, No. 4 = 10,000,000, No. 5 = 100,000,000, No. 6 = 1,000,000,000 bacteria per ml.). No. 8 is 0.005 ml. of distilled water bacterized to the consistency of heavy cream. Resistant cysts represented the end result in all dilutions except No. 8, where death occurred.

FIG. 2.—Graphic representation of data contained in Table 1. All points represent averages of 30 vials in 3 successive experiments. Constant volume (0.5 ml.), varying bacterial concentrations (1 = 637,000 per ml.; 2 = 6,370,000 per ml.; 3 = 63,700,000 per ml.; 4 = 637,000,000 per ml.; 5 = 6,370,000,000 per ml.; 6 = 63,700,000,000 per ml.; 7 = 637,000,000,000 per ml.).

heavy suspension of *Aerobacter* (average concentration, for three separate experiments, being 637 billion per ml. viable count). These series were numbered 1–7 from the greatest dilution to the 1:1 series (637 billion), respectively (see legend of Fig. 2). Into each vial

(containing 0.5 ml. of suspension) a single trophic ciliate was introduced. Counts of the numbers of ciliates present at the end of 24 hours were made, and the end result of growth was determined at 72 hours. These results are presented in Table 1 and are graphically represented in Figure 2. In dilutions 1, 2, and 3 there was an increasing difference of reproductive rates at the end of 24 hours. Dilution 4 showed less growth than 3, and dilution 5 was still lower. In most of the vials of dilution 6 the ciliates died, and in the remaining few reproduction was negligible. No ciliate survived in dilution 7, although this concentration was lower than our "heavy cream" bacterized hanging drops. After 72 hours the vast majority of ciliates in dilutions 1 through 5 had encysted, and the fluid was crystal clear. Counts of these end results showed that as the number of available bacteria was increased the number of ciliates produced in a given period of time was greater. In one vial of series 5, 702,000 ciliates represented the end result of the growth of a single cell, and the average of all the vials of that series was 457,620. Series 6 showed only three vials containing living ciliates and no cysts, with the low average count of 1,616.

TABLE 1

0.5 ML. VOLUME; VARYING BACTERIAL CONCENTRATIONS

Dilution Number	Bacteria per Ml.	Ciliates at 24 Hours	Ciliates at 72 Hours	Maximum Number Obtained
1	637,000	2,038	3,906	4,940
2	6,370,000	3,782	7,338	12,090
3	63,700,000	10,070	19,500	22,740
4	637,000,000	8,182	61,534	80,900
5	6,370,000,000	4,190	457,620	702,000
6	63,700,000,000	5 (1 vial)	1,616 (3 vials)	4,850
7	637,000,000,000	0	0	0

A number of facts were suggested by these results. If there is sufficient space for growth, the number of ciliates capable of being produced from a single cell in a given period of time varies directly with the number of food organisms present, up to a certani concentration for that volume. The reproductive rate also increases as the food increases up to a certain bacterial concentration. Also the growth-rate of the ciliates is lower in the early stages of the high concentrations, but, as the number of ciliates increases, the growth-rate becomes accelerated so that the peak of the curve in Figure 1 is at dilution 3 at 24 hours but has shifted to dilution 5 at 72 hours. Because concentrations 6 and 7 are lower than our highest concentrations in the hanging drops, it appears that there is a volume effect in these experiments. It must be admitted that physical differences between the vial and hanging-drop fluids might conceivably produce different results which would invalidate any direct comparisons between the two. Comparisons may be made, however, between different concentrations of bacteria in either drops or vials.

VARYING VOLUMES OF A SINGLE FOOD CONCENTRATION

Our next group of experiments was designed to test the effect of varying volumes with a single bacterial concentration. Concentration 7 (637 billion per ml.), in which no single ciliate had lived in the 0.5 ml. volumes, was chosen. Five different volumes were set up

in series of ten vials each, the experiment repeated on three successive occasions. The volumes chosen were 0.01 ml., 0.02 ml., 0.04 ml., 0.08 ml., and 0.16 ml. A single ciliate was introduced into each vial. Counts were made at the end of 36, 48, 60, 72, and 84

TABLE 2

CONSTANT BACTERIAL CONCENTRATION (DIL. NO. 7); VARYING VOLUMES

Volume	Number of Ciliates per 0.5 Ml.					
	36 Hours	48 Hours	60 Hours	72 Hours	84 Hours	Maximum No.
.01 ml.	.....	.....	669,800	1,090,200	.....	1,680,000
.02 ml.	5,707	27,900	734,500	1,133,000	1,753,600	2,200,000
.04 ml.	3,520	24,700	426,500	1,440,000	1,937,400	2,550,000
.08 ml.	1,300	5,300	23,125	49,330	Dead	204,000
.16 ml.	60 (1 vial)	0	0	0	0	.....

hours, except in the case of the 0.01 ml. volumes. The results of these counts are presented in Table 2 and in Figure 3.

Whereas no ciliates survived in this bacterial concentration in the 0.5 ml. volumes in the preceding experiment (Table 1), there was some survival in all the series from 0.01 ml. to 0.16 ml., although in the last series only one vial out of thirty showed growth, and that extremely low.

The smaller volumes showed a higher division-rate through the first 60 hours of growth, but the numbers of ciliates in the 0.04 ml. vials were greater, though only slightly so, at 72 and 84 hours. The relatively small difference in numbers is due to the fact that the end point (encystment) was reached at 72 hours in the 0.01 ml. vials and slightly later in the 0.02 ml., but in the 0.04 ml. vials encystment was not complete even at 84 hours. In the 0.08 ml. volumes the depressing effects noted in the previous experiment occurred, with slow growth up to 72 hours and then rapid death without encystment. Volumes of 0.16 ml. almost completely suppressed growth.

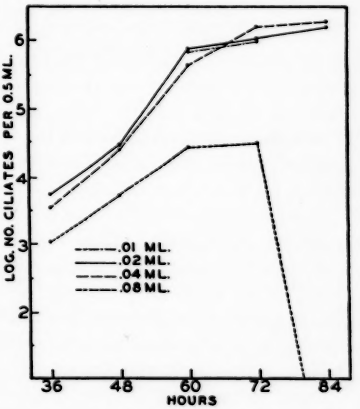

FIG. 3.—Graphic representation of data contained in Table 2. All points represent averages of 30 vials in 3 successive experiments. Varying volumes (0.01 ml.–0.08 ml.), constant bacterial concentration (No. 7 of Table 1, 637,000,000,000 *Aerobacter* per ml.).

DISCUSSION

We have shown in Part I of this series (Kidder and Stuart, 1939) that many different types of bacteria may exert a growth-limiting influence on a population of *Colpoda* because of their inferior qualities as food or because of their toxicity. When these types are removed and only *Aerobacter* (an excellent food organism) is presented, there occurs an enormous population increase in proportion to the

concentration of bacteria, within limits. Some of the factors limiting growth under these conditions appear to be the available bacteria, the available space for growth, and the depressing effect of high bacterial concentrations in large volumes. The latter may be due to oxygen deficiency, as has been shown to be the case by Phelps (1936) and Taylor and Strickland (1938) working with larger volumes. In separate experiments we have been able to obtain growth in aeration flasks using larger volumes and heavier bacterial concentrations than could be used without aeration. In the small volumes, however, it appears that the oxygen-tension factor may be of less importance, owing to the large surface area exposed.

Contrary to the earlier views of Woodruff (1911) and a number of others since then, our results show that excretory substances appear to play only a small rôle in growth limitations of *Colpoda*. This was also the conclusion reached by Phelps (1936) in dealing with bacteria-free cultures of *Glaucoma*.

Crowding—a term applied to conditions where it is thought that the volume of the animals contained in a given space becomes too large for compatibility—operates in the smallest volumes (0.005 ml.) of *Colpoda*, tending to limit and stop their growth. But the crowding here is an actuality not an effect produced by a few organisms in a large volume. The ciliates become so concentrated that they are in actual physical contact with one another. When this condition is reached, the ciliates rupture. We realize that by pointing to physical contact as a limiting factor we have given no explanation but only a description of what happens.

In our opinion a very important factor in the growth limitation of a population of *Colpoda* is the quantity of available food. That other factors enter in, to a lesser degree, we do not deny. Temperature certainly plays a part. Hydrogen-ion concentration may play a small part, but not an appreciable one between the limits of pH 5 and pH 8.5. Decrease in oxygen tension may play a part, especially in larger volumes. Concentration of metabolic waste products of the protozoa or of the food organism is apparently ineffective, otherwise their effects would be seen by the time the ciliates reached the concentration of 20,000 per 0.005 ml.

It seems apparent that the same factors operating to limit growth may influence encystment of *Colpoda*. Dilution 8 ("heavy cream") of Figure 1 shows a concentration equal to approximately 4 million ciliates per ml., and yet no cysts were formed. Thus the view that encystment is induced by an accumulation of metabolic waste products of the ciliates themselves apparently does not apply in the case of this strain of *Colpoda*. This is also the conclusion reached by Taylor and Strickland (1938) in the case of *C. duodenaria*, although it was stated otherwise in an earlier report (Barker and Taylor, 1931).

From our observations we believe that encystment in our strain of *Colpoda* is brought about, under the conditions of our experiments, principally by the lack of available foods and that "crowding" does not result in encystment but in death.

The results here reported do not answer all the questions relating to the interesting question of growth limitations in populations of *Colpoda* but simply point out some of the possibilities under experimental conditions.

SUMMARY

1. The potentialities of growth of a strain of the ciliate *Colpoda* have been investigated.

2. The food used was the coliform bacterium, *Aerobacter*, suspended in distilled water.

3. Single ciliates when introduced into varying bacterial concentrations reproduced in direct relation to the bacterial count.

4. Single ciliates introduced into varying volumes (from 0.01 to 0.16 ml. in vials) of a known bacterial concentration grew readily and to great concentrations in the smaller volumes but practically not at all in the larger volumes.

5. It was concluded that the available food is one of the principal factors operating to limit the population of this ciliate.

6. Encystment appears to be the result of the depletion of the available food, for in no case did it take place when viable *Areobacter* were present in quantities.

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GROWTH STUDIES ON CILIATES. III. EXPERIMENTAL ALTERATION OF THE METHOD OF REPRODUCTION IN COLPODA[1]

(One figure)

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IN ALL species of the genus *Colpoda* reproduction normally takes place within a cyst wall. This wall is secreted by the ciliate during the early phases of mitotic activity and is accompanied by a rounding-out of the cell. Within the division cyst two daughter-cells are produced, and these divide again to produce four cells. There is a gradual redifferentiation of the characteristic morphological structures of the trophic ciliate in each daughter, swimming activities become more and more pronounced until the cyst wall ruptures at one point, and the four daughter-ciliates escape one by one and swim away. On rare occasions two or eight daughter-cells are produced within the division cyst and these escape from the cyst as do the four. This mode of division has been described in detail for a number of species of *Colpoda* and certainly represents the normal method of reproduction.

Alterations in the foregoing method of reproduction do occur, however. Penn (1937) describes quadruple division of *Colpoda cucullus* without the formation of a cyst wall. He is inclined to think that cyst formation is an adaptation on the part of the ciliate to an adverse environment and that quadruple division without a cyst wall represents the normal method of reproduction when the conditions are optimum for growth. This belief has been criticized in regard to *C. cucullus*, since during long-continued cultivation of this species in boiled hay only rare cases of division without a cyst wall occurred (Kidder and Claff, 1938).

Under certain conditions, *Colpoda* sp.[2] reproduces without a cyst wall. Various other modifications of the normal division-cyst method of reproduction also occur. The conditions under which these departures from the normal make their appearance have been investigated and are the subject for the present report. A few new terms, descriptive in nature, have been introduced.

DESCRIPTION OF THE DIFFERENT TYPES OF REPRODUCTION

In view of the fact that the alterations in the method of reproduction form the basis for what is to follow, a short description of their morphological differences will be given. We have designated the alterations (from the normal "division cyst, *DC*") as follows: "atypical division cyst" (*ADC*), "fission-division cyst" (*FDC*), "fission division" (*FD*) and "simple fission." Other alterations have been noted, but they do not enter into the present discussion.

Atypical division cyst.—This modification of the normal type of reproduction involves

[1] This investigation was supported in part by a grant-in-aid from the Society of the Sigma Xi.

[2] The strain of Colpoda used in this investigation is the same as that reported in the first paper of this series (Kidder and Stuart, 1939*a*).

the later stages of the reproductive phase, and it is only at the excystment of the four daughter-cells that it can be detected. During reproduction by normal division cysts, after the four daughters have redifferentiated within the cyst wall, they become very active and resemble in every detail trophic ciliates. When the cyst wall finally ruptures at some point, the ciliates emerge with almost explosive suddenness and immediately swim away. In the atypical division cyst the development of four daughter-ciliates parallels the normal until almost ready to excyst. They are never as perfectly shaped or as active, however, and when the cyst wall ruptures they squeeze out through the resulting opening

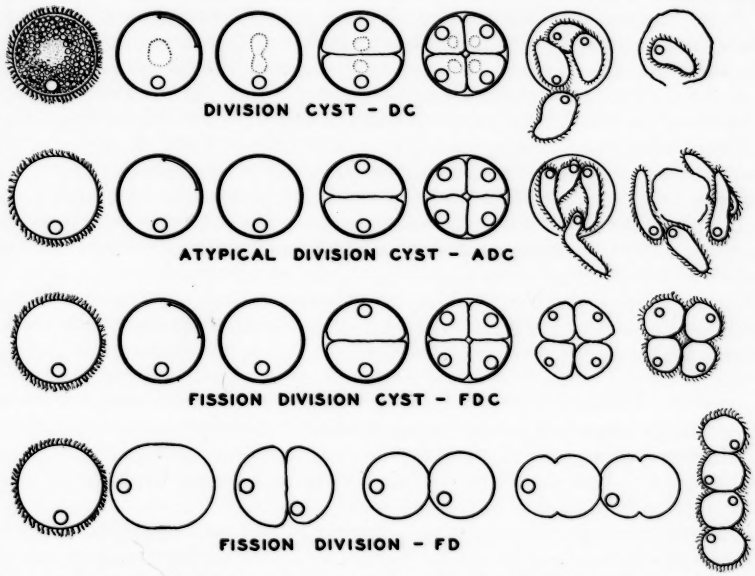

FIG. 1.—Diagrammatic representation of various methods of reproduction in *Colpoda*. Within the cells the dotted circles represent the macronuclei, while the solid circles represent the contractile vacuoles; arrows indicate rotation.

one by one and very slowly. They are always elongated "wormy" forms and do not swim away but remain in the immediate vicinity of the old cyst wall for some time (Fig. 1, atypical division cyst, *ADC*). Eventually they develop normal activity and are indistinguishable from any normal young trophic form.

Here it would seem that the redifferentiation of the daughter-cells is completed outside the cyst wall, since the wall appears to be weak and prematurely ruptures.

Fission division cysts.—Slightly more radical than the foregoing modification is the fission-division cyst. The early stages of this reproductive process are identical with the normal and with the atypical division cyst, but after the four-cell stage is reached and before redifferentiation of the characteristics of the trophic form occurs, the cyst wall ruptures and disappears. This leaves the four daughter-cells exposed to the environment (Fig. 1, fission-division cyst, *FDC*). Slow redifferentiation takes place and finally the four round,

feebly moving ciliates become somewhat separated. Eventually each attains the normal form and swims away.

In this modification the cyst wall is weaker and less resistant than in the previous one, and shortly after it ruptures no trace of it can be seen.

Fission division.—This type of reproduction is accomplished without the formation of a cyst wall. As the ciliate prepares for reproduction it rounds up in the normal manner. The revolving motion, so noticeable in the early stages of the normal division cyst, is entirely lacking. For that reason fission division can be detected in the very early phases. The cilia appear to be partially withdrawn, and motion ceases. Slight elongation takes place during the period of macronuclear division, and the first plane of fission ensues. The two daughter-cells become quite round but do not separate. Each daughter elongates and divides, the planes of their division being parallel to the first fission plane instead of at right angles to it, as in the division cyst. This results in a characteristic chain of four cells, all rounded and retaining contact with each other (Fig. 1, fission division, *FD*). The cilia become active, and the whole chain vibrates slowly. Eventually, the four daughters separate, slowly attain the normal form, and start swimming.

It should be noted, in relation to the second fission plane, that its direction, parallel to the first, is apparent but not real. Before the second fission starts there is rotation on the part of the first two daughters, so that the polarity is probably not different from the normal division-cyst daughters.

This type of reproduction appears to be approaching the condition found in the vast majority of ciliates where no cyst is formed. It is characteristically quadruple fission, although occasionally only one division occurs.

Simple fission.—Under certain conditions, rare cases of irregular binary fission have been observed. Elongation of a swimming trophic ciliate occurs, and eventually two daughter-ciliates result. The fission plane is somewhat oblique to the long axis of the body. The body outlines of both daughters are irregular and appear somewhat abnormal, but when isolated these daughters are found capable of normal growth, reverting immediately to the division-cyst type of reproduction.

With the occurrence of simple fission we see that this ciliate is capable of altering its method of reproduction from its normal highly specialized division cyst, through a series of changes, to the binary fission type characteristic of most ciliates.

EXPERIMENTAL PROCEDURES AND RESULTS

On January 28, 1938, a 5″×$\frac{5}{8}$″ culture tube containing 6 ml. of sterile distilled water was bacterized with a 24-hour agar slant culture of *Aerobacter cloacae*. In all experiments herein reported this species was used exclusively for bacterization purposes. Several *Colpoda* (Kidder and Stuart, 1939*a*) from a hay infusion culture were inoculated into the bacterial suspension. A week later this tube was rebacterized with approximately the same amount of bacteria. At this time the usual heavy ring of resting cysts had formed on the tube at the surface of the liquid. Thereafter, the tube was rebacterized at 7- to 10-day intervals, and sterile distilled water added to keep the contents of the tube at about the same level. By the third or fourth bacterization the ring of resting cysts had completely disappeared, and so far as could be ascertained resting cysts did not again form in this tube during the course of the experiment, which continued for approximately 10 months.

The ciliates originally inoculated into the experimental tube carried over some of the

bacterial flora of the hay infusion. It seemed of interest to determine whether the extraneous bacteria would survive the frequent massive inoculations of *Aerobacter*. Survival had been shown to occur in 48-hour serial transplants (Kidder and Stuart, 1939*a*). Accordingly, flooded plates were prepared at different intervals throughout the experiment from suitable dilutions of the suspension from the experimental tube. These plates indicated that the proportions of the extraneous bacterial flora remained relatively constant throughout the experiment. Four to five days after bacterization with *Aerobacter* the organisms present in the tube were roughly estimated as 80 per cent *Pseudomonas* (it should be noted that while the diffusible pigment of the *Pseudomonas* organisms was very marked on the agar plates it was never observed in the experimental tube), 15 per cent *Flavobacterium*, and small numbers of *Aerobacter*, *Achromobacter*, *Bacillus*, *Clostridium*, and other undetermined genera.

At irregular intervals bacterized hanging drops containing from 3 to 5 ciliates were prepared from the liquid in the experimental tube. As a control similar preparations were made from a 24-hour tube of our *Colpoda-Aerobacter* pure series (Kidder and Stuart, 1939*a*). In this way the growth of the ciliates could be observed in a medium in which growth products had accumulated for only 24 hours, as well as in a medium in which growth products were constantly accumulating. The speed of growth and number of divisions in the experimental and control hanging-drop preparations were approximately equal for 6 months. After 6 months, growth of ciliates in the experimental tube steadily declined. The growth of experimental ciliates inoculated into hanging drops of bacterized distilled water also steadily declined. In October, 1 ml. of the contents of the experimental tube had to be concentrated by centrifugation in order to obtain ciliates, while in November, approximately 10 months after the start of the experiment, no ciliates were found when the entire contents of the tube was concentrated.

From January 28 to May 1 no change in the method of reproduction was noted in the ciliates from the experimental tube. On June 28, however, hanging drops prepared from the experimental tube showed that 100 per cent of the ciliates were dividing by fission division. Control preparations of normal ciliates from S 42 (the forty-second transplant of ciliates which had been sterilized and were being cultivated on pure *Aerobacter*) showed only regular division cysts.

On June 30 both the ciliates and the ciliate-free medium of the experimental tube were studied. One milliliter of the medium was centrifuged at 2,000 r.p.m. for 6 minutes to concentrate the ciliates. About 0.75 ml. of the supernatant fluid was then transferred to a second tube and centrifuged at high speed to remove all ciliates. The supernatant fluid from this second tube was saved, and the centrifugate discarded. To the concentrated ciliates in the first tube 10 ml. of distilled water were added, and the tube centrifuged again at low speed. Approximately 9.5 ml. of the supernatant fluid were discarded and 9.5 ml. of distilled water added. This procedure was repeated five times to dilute out accumulated growth products.

Unless otherwise specified all experiments were set up in duplicate.

Loopfuls of the ciliate-free supernatant fluid from the experimental tube were heavily, moderately, and lightly bacterized, single washed ciliates from the experimental tube were added to these suspensions, and the cover glasses inverted and sealed onto depression slides. This procedure was repeated with the washed ciliates in distilled water bacterized as above. Washed normal ciliates from S 43 were set up in the same way in the supernatant fluid of the experimental tube and also in distilled water. The results of these experiments are recorded in Table 1.

TABLE 1

WASHED CILIATES FROM THE EXPERIMENTAL TUBE ON JUNE 30 IN BACTERIZED EXPERIMENTAL SUPERNATANT FLUID

Bacterization	Experiment	Type of Reproduction*
Heavily	1	*FD* 7*G*—complete rupture
	2	*FD* 7*G*—complete rupture
Moderately	1	*FD* 6*G*—*DC* 7*G*—*RC* many degenerate
	2	*FD* 5*G*—*DC* 6*G*—*RC*
Lightly	1	*FD* 3*G*—*DC* 4*G*—*RC*
	2	*FD* 3*G*—*DC* 4*G*—*RC*

WASHED EXPERIMENTAL CILIATES IN BACTERIZED DISTILLED WATER

Bacterization	Experiment	Type of Reproduction*
Heavily	1	*FD* 7*G*—complete rupture
	2	*FD* 7*G*—complete rupture
Moderately	1	*FD* 5*G*—*DC* 6*G*—*RC*
	2	*FD* 5*G*—*DC* 6*G*—*RC*
Lightly	1	*FD* 4*G*—*DC* 5*G*—*RC*
	2	*FD* 3*G*—*DC* 4*G*—*RC*

WASHED NORMAL CILIATES IN BACTERIZED EXPERIMENTAL SUPERNATANT FLUID

Bacterization	Experiment	Type of Reproduction*
Heavily	1	*FD* 7*G*—complete rupture
	2	*FD* 7*G*—complete rupture
Moderately	1	*FD* 6*G*—*DC* 7*G*—*RC* some degenerate
	2	*FD* 5*G*—*DC* 6*G*—*RC*
Lightly	1	*FD* 3*G*—*DC* 4*G*—*RC*
	2	*FD* 4*G*—*DC* 5*G*—*RC*

WASHED NORMAL CILIATES IN BACTERIZED DISTILLED WATER

Bacterization	Experiment	Type of Reproduction*
Heavily	1	*DC* 7*G*—*RC*
	2	*DC* 7*G*—*RC*
Moderately	1	*DC* 6*G*—*RC*
	2	*DC* 6*G*—*RC*
Lightly	1	*DC* 4*G*—*RC*
		DC 4*G*—*RC*

* *FD* = Fission Division; *RC* = Resting Cyst; *DC* = Division Cyst; *G* = Generation.

Example: *FD* 6*G*—*DC* 7*G*—*RC* many degenerate. Ciliate reproduced by fission division for six generations, by division cysts in the seventh generation. Resting cysts were then formed, many of which were degenerate.

Table 1 shows that ciliates from the experimental tube in both heavily bacterized supernatant fluid and distilled water reproduced for seven generations by fission division then ruptured. In the moderately bacterized suspensions the ciliates reproduced by fission division for five to six generations, then for one generation by division cysts, finally forming resting cysts. In the lightly bacterized preparation fission division extended to the third or fourth generation, followed by one generation of division cysts, before resting cysts were formed. It will be noted that where the concentration of food is not too heavy a division cyst always intervenes between fission division and resting cysts. Over 500 such preparations have been followed, and in no case have resting cysts been observed to form immediately after a generation of fission division. In several experiments cover slips were removed, and fresh unbacterized supernatant fluid added at the fourth or fifth division; nevertheless, a generation of division cysts occurred before resting cysts were formed. It cannot be said that occasional individual ciliates do not form resting cysts immediately after fission division, since, where we are dealing with hundreds or thousands of ciliates in one drop, it is impossible to follow each ciliate. Ciliates in unbacterized experimental supernatant fluid produced one or two generations by fission division, then a generation by division cysts followed by resting cysts. The total number of ciliates is sufficiently small that it can be safely said that no resting cysts formed except after a generation of division cysts. Even washed fission-division forms in unbacterized distilled water produced one generation by division cysts before going into resting cysts.

Washed normal ciliates in bacterized experimental supernatant fluid reproduced by fission division just as did the experimental ciliates. On the other hand, normal ciliates in bacterized distilled water produced only regular division cysts. This would seem to indicate that the medium in the experimental tube had been altered or conditioned by the growth of the ciliates, by the growth of the bacteria, or by both. For convenience the term "conditioned medium" will be substituted hereinafter for "the supernatant fluid from the experimental tube," and, when referring to what seems to be some substance or substances in the conditioned medium responsible for certain reactions, the term "conditioning factor" will be used.

There were at this time (June 30), 21,000 ciliates per milliliter in the experimental tube. Distilled water was added to the experimental tube, and bacterization continued until November 16.

On July 28, August 28, October 4, and November 16, the reaction of normal and experimental ciliates to the conditioned medium, the species specificity of the conditioned medium, the nature of the conditioned medium, and other factors were studied. The results of these experiments will be discussed under various headings.

Normal ciliates in conditioned medium.—Normal ciliates from S 55 in moderately bacterized conditioned medium on July 28 produced, as in the previous month, 100 per cent fission division for five to six generations, then one generation of division cysts followed by resting cysts. Ciliates from S 55 in bacterized distilled water reproduced by division cysts. The rate of division of the normal ciliates in the conditioned medium was slower than in the distilled water and, moreover, small numbers of degenerate resting cysts were formed in the conditioned medium, and only normal resting cysts in the distilled water.

The same results were obtained with ciliates from S 68 on August 28 and from S 85 on October 4, except that the rate of division in conditioned medium was progressively slower than the controls in distilled water. Ciliates in the conditioned medium of October 4 formed, for the most part, degenerate cysts.

On November 16, ciliates from S 104 in the conditioned medium reproduced by fission division for only two to three generations. After that division cysts, fission-division cysts, fission division, and atypical division cysts all occurred in some hanging drops. While these ciliates produced from six to seven generations in 96 hours, the trophic forms ruptured without forming resting cysts. Thus the conditioning factor which was first found in the experimental tube on June 28 was present but only in weak concentration on November 16. The fact that no ciliates were found in the experimental tube at this time may account for the weakness of the conditioned medium. The controls, ciliates in S 104 in bacterized distilled water, produced six to seven generations by division cysts in 48 hours, and formed normal resting cysts.

It was noted that the first division of a normal ciliate in conditioned medium might be either by division cysts or by fission division. The type of division seemed to be determined by the time elapsing before division. Ten normal ciliates immediately after excystment, ten ciliates 2 hours after, and ten ciliates 3.5 hours after excystment were individually placed in bacterized conditioned medium. The first ten ciliates (immediately after excystment) all reproduced by fission division. Six of the next group reproduced by fission division and four by division cysts. All of the last group reproduced by division cysts. The succeeding five or six generations in every case were produced by fission division.

To determine the effect of continued growth of a normal ciliate in conditioned medium, duplicate hanging-drop preparations were made, on July 28, of ciliates from S 57 in bacterized conditioned medium. The ciliates reproduced by fission division for six generations, then one generation of division cysts followed by resting cysts. Just before resting cysts were formed, a ciliate was isolated from each preparation and again set up in conditioned medium. This procedure was repeated ten consecutive times. Each transfer produced results wholly comparable to the first setup. The ciliates from the tenth transfer, before forming resting cysts, were washed five times in distilled water by centrifugation, and the isolated ciliates set up in bacterized distilled water. These ciliates reproduced the same as they had in conditioned medium. They were again washed and set up in bacterized distilled water. Fission division occurred for only three generations, followed by one generation of mixed fission-division and atypical division cysts, two generations of fission-division cysts and division cysts, and one generation of division cysts only. A third transfer of washed ciliates produced only division cysts.

It would seem from this that a normal ciliate growing in conditioned medium for approximately sixty-five generations continues to reproduce by fission division for several more generations when transferred to bacterized distilled water. It will be shown later that the conditioned medium of July 28, when diluted ten times, did not cause fission division in normal ciliates. Since the material from the tenth transfer was diluted more than a million times by washing, it is improbable that sufficient conditioned medium could have been carried over into the bacterized distilled water to cause fission division. It is also difficult to conceive of sufficient conditioned medium, adsorbed on the surface of the isolated ciliate, being carried over to the distilled water to cause fission division for seven generations.

Ciliates from the experimental tube in distilled water and in conditioned medium.—On June 28, the first time the conditioning factor was found in the experimental tube, washed single ciliates from this tube, in bacterized distilled water, reproduced by fission division for five to six generations, then by division cysts for one generation, followed by resting cysts. Washed ciliates from the same tube on July 28 showed marked variation in meth-

ods of reproduction in bacterized distilled water (Table 2). The ciliates in Experiment 4 (Table 2) reacted as had all the ciliates the previous month. The ciliates in Experiment 8 reacted in reverse fashion, producing five generations of division cysts, one of fission division, and finally a generation of division cysts. On the other hand, ten washed ciliates from the experimental tube in bacterized conditioned medium produced only fission-division forms for five or six generations, followed by the usual generation of division cysts.

Serial transfers of the ciliates in Experiments 4 and 8 (Table 2) to bacterized distilled water were carried out in the manner previously described. Ciliates from Experiment 4 continued to reproduce by fission division to about the sixth generation for six consecutive transfers, to the fourth generation on the seventh, and to the second generation on

TABLE 2

WASHED CILIATES FROM THE EXPERIMENTAL TUBE ON JULY 28 IN BACTERIZED DISTILLED WATER

Experiment	Type of Reproduction*
1.....	*FD* 4*G*—*ADC*+*FDC*+*DC* 5*G*—*DC* 6*G*—*RC* many degenerate
2.....	*FD* 5*G*—*DC* 6*G*—*RC*
3.....	*FD* 5*G*—*DC* 6*G*—*RC*
4.....	*FD* 6*G*—*DC* 7*G*—*RC* many degenerate
5.....	*DC* 3*G*—*ADC* 4*G*—*FDC* 5*G*—*FD* 6*G*—*DC* 7*G*—*RC* many degenerate
6.....	*FD* 4*G*—*DC* 7*G*—*RC*
7.....	*FD* 7*G* complete rupture
8.....	*DC* 5*G*—*FD* 6*G*—*DC* 7*G*—*RC* many degenerate
9.....	*FD* 3*G*—*FDC*+*ADC*+*DC* 5*G*—*DC* 6*G*—*RC* few degenerate
10.....	*FD* 6*G*—*DC* 7*G*—*RC*

* *DC* = Division Cysts; *ADC* = Atypical Division Cysts; *FDC* = Fission-Division Cyst; *FD* = Fission Division; *RC* = Resting Cyst; *G* = Generation.

Example: *FD* 4*G*—*ADC* + *FDC* + *DC* 5*G*—*DC* 6*G*—*RC* many degenerate. Ciliates reproduced by fission division for four generations. In the fifth generation some of the ciliates reproduced by atypical division cysts, others by fission-division cysts, and some by division cysts. In the sixth generation all ciliates reproduced by division cysts. Resting cysts were then formed, many of which were degenerate.

the eighth transfer. The next transfer showed only regular division cysts. Ciliates from Experiment 8 reproduced by division cysts for two generations in the second transfer, then by fission division to the sixth generation. In the next three transfers reproduction was by fission division with a return to reproduction by division cysts on the next transfer. Thus after a month or more in conditioned medium, several consecutive transfers in bacterized distilled water were required before normal division cysts were again produced. We cannot account for the fact that the ciliates in Experiment 8 first produced division cysts, followed by fission division, which in turn were followed by division cysts.

On August 28, washed ciliates from the experimental tube were again set up in bacterized distilled water and in bacterized conditioned medium. The ciliates in the conditioned medium reproduced by fission division as in previous experiments. The reactions of the ciliates in distilled water are shown in Table 3. All the ciliates reproduced by division cysts for at least four generations. Some then produced fission-division forms, others did not. A ciliate from Experiment 1 (Table 3) was carried through two transfers in

bacterized distilled water. In both transfers reproduction was by fission division to the fifth or sixth generation. A ciliate from Experiment 3 reproduced by division cysts for two consecutive transfers. In the third transfer reproduction occurred by division cysts

TABLE 3

WASHED CILIATES FROM THE EXPERIMENTAL TUBE ON AUGUST 28 IN BACTERIZED DISTILLED WATER

Experiment	Type of Reproduction*
1.....	*DC 4G—ADC+FDC+FD 5G—FD 6G—DC 7G—RC*
2.....	*DC 5G—ADC+FD 6G—DC 7G—RC*
3.....	*DC 7G—RC*
4.....	*DC 5G—ADC+FD 6G—DC 7G*—complete rupture
5.....	*DC 6G—DC+ADC+FD 7G—RC*+rupture+degenerate *RC*
6.....	*DC 7G—RC*
7.....	*DC 5G—FD 6G—DC 7G—RC* some degenerate
8.....	*DC 6G—RC*
9.....	*DC 5G—ADC+FD 6G—FD 7G* rupture
10.....	*DC 5G—ADC+AFD+FD 6G—DC 7G—RC*

* For explanation see Table 2.

to the third generation, then by fission division to the sixth generation. Reproduction by fission division for five generations took place in the fifth transfer.

Washed ciliates from the experimental tube in bacterized conditioned medium reproduced by fission division on October 4. The reactions of ciliates in bacterized distilled

TABLE 4

WASHED CILIATES FROM THE EXPERIMENTAL TUBE ON OCTOBER 4 IN BACTERIZED DISTILLED WATER

Experiment	Type of Reproduction*
1.....	*DC 5G—DC+FD 6G—DC 7G* rupture+degenerate *RC*
2.....	*DC 6G—RC*
3.....	*DC 7G—RC* many degenerate
4.....	*DC 5G—DC+FD 6G—FD 7G* rupture
5.....	*DC 7G—RC* many degenerate
6.....	*DC 7G—RC* few degenerate
7.....	*DC 6G—RC*
8.....	*DC 6G—RC*
9.....	*DC 7G—RC* many degenerate
10.....	*DC 7G—RC* few degenerate

* For explanation see Table 2.

water are recorded in Table 4. It will be noted in this table that only ciliates 1 and 4 produced any fission-division forms. Ciliates from Experiments 2, 7, and 10 were carried through seven transfers. Growth of the ciliates was increasingly poorer in the second, third, and fourth transfers. An average of only four generations was produced in the fourth transfer. Small numbers of fission-division forms (not more than 10 per cent)

were produced in each series in the fourth or fifth generation of the third to fifth transfers. All other reproduction was by regular division cysts.

It has been shown that washed ciliates from the experimental tube on June 28 reproduced by fission division in bacterized distilled water. On July 28 some of the ciliates from the same tube reproduced by fission division, others by division cysts. The latter reproduced by fission division in subsequent transfers in bacterized distilled water. All washed ciliates from the experimental tube on August 28 produced only division cysts for from four to seven generations. Selected ciliates in serial transfers reproduced entirely by fission division in one or another transfer. On October 4 only two of the ten ciliates tested reproduced by fission division. Serial transfers of those producing only division cysts produced no more than 10 per cent fission division in any generation of any transfer. Had ciliates been found in the experimental tube on November 16, it is possible that they would have failed to reproduce by fission division under any condition. At the present time no explanation for these facts is apparent.

Species specificity to the conditioned medium.—To test the species specificity of the conditioned medium, fifteen wild *Colpoda* were isolated from several infusions of soil or grass in water. These ciliates were individually transferred to tubes containing 6 ml. of bacterized distilled water. After they had become acclimatized and had multiplied in this environment, single ciliates (in duplicate preparations) were placed in hanging drops of bacterized distilled water and in bacterized conditioned medium. Nine of the strains reproduced by division cysts, in the conditioned medium and in distilled water. Five serial transfers of these ciliates were made in bacterized conditioned medium without any change in their method of reproduction. Upon structural analysis all these nine strains were found to differ from our stock strain of *Colpoda*.

Ciliates from the six remaining cultures, Nos. 3, 6, 7, 8, 9, and 12 in bacterized conditioned medium reproduced by fission division for five or six generations as did our stock strain from S 59. In bacterized distilled water all, with the exception of strain 3, reproduced only by division cysts. Strain 3 in the fifth generation showed atypical division cysts and two fission-division forms. These six wild strains and ciliates from S 59 were transferred serially for from four to seven transplants in bacterized distilled water. (It should be pointed out that where a number of strains were to be transferred into bacterized distilled water or conditioned medium, the required amount of liquid was bacterized before loops of the material were placed on the cover slip to receive the ciliate, in order to insure equal amounts of food.) Strain 3 showed about 10 per cent fission division in the fourth and fifth generations of the second transfer; about 20 per cent in the third to fifth generations of the third transfer; and about 50 per cent in the fourth generation of the fourth transfer. At this point all the ciliates of this strain ruptured. Strains 6, 7, 8, 9, 12, and S 59 showed only division cysts in seven transfers, but marked variation in the reproduction rate and the total number of divisions were noted. Stock ciliates from S 59 and strains 7 and 9 produced from six to seven generations in 48 hours, culminating in normal resting cysts. Strain 6 produced only four to five generations in 72 hours with many degenerate resting cysts. Strains 8 and 12 produced five to six generations in 48 hours with normal resting cysts.

Strains 6, 7, 8, 9, and 12 were found to be identical with our stock strain of *Colpoda* on the basis of the number of ciliary rows, position and shape of the mouth, and type of nuclei, both resting and fission. Strain 3, although the ciliary lines, mouth, and macronucleus are identical with our stock, differs in one important detail. The micronucleus is

extremely small. The number of chromosomes appears to be large, while eight is the number for our stock strain, (The evidence for this will be reported at a later date.)

These strains have been carried in the laboratory for 5 months. Strains 8 and 12, in the usual 6 ml. of bacterized distilled water, produced in 48 hours a heavy ring of cysts about the tube; strains 7 and 9 grew well but sometimes failed to encyst; while Nos. 3 and 6 frequently had to be transferred to 0.5 ml. of bacterized distilled water in a small vial (Kidder and Stuart, 1939*b*) to insure maintenance of the strains. It is evident from this that not all strains of the same species of *Colpoda* are alike in their growth potentialities, and as is the case with bacteria, one should hesitate before assuming that the reactions obtained with a single strain of a species will necessarily hold true for all strains of that species even under identical conditions.

Reactions of a modified strain of the stock Colpoda.—In the course of sterilizing cultures of *Colpoda* as previously described (Kidder and Stuart, 1939*a*) loops of the washed ciliates were inoculated into tubes containing 6 ml. of sterile broth as a check on sterility. One such tube which showed no evidence of growth after 7 days was reincubated. After 14 days a ring of resting cysts was noted, but the broth appeared clear. The broth was plated out and found to contain a pure culture of a *Mycobacterium* which requires 9 days for the production of pin-point colonies. As the ciliates were growing in a pure culture of the *Mycobacterium*, transplants were made at 2- or 3-week intervals into broth and the culture designated as *M* ciliates. It has been since found that this represents an almost ideal way for carrying this strain of *Colpoda*. No bacterization is necessary, the bacteria never overgrow the ciliates, and transplants at 2- or even 3-month intervals are all that are required.

The *M* series was carried in a special rack, and old transplants were not discarded. The first transplant was made on March 26. By August 28 the broth had evaporated to less than 1 ml., yet there was a large number of very small trophic ciliates in the medium. On that date the usual hanging-drop preparations in bacterized conditioned medium and bacterized distilled water were made from the *M* ciliates of the March 26 and the August 12 tubes.

Ciliates from the March 26 tube reproduced very slowly by division cysts in both conditioned medium and distilled water. After the fourth generation the ciliates were large and filled with vacuoles. After five generations all the ciliates ruptured. Before all had ruptured, however, some ciliates from the conditioned medium were transferred to fresh bacterized conditioned medium, and some from distilled water to fresh bacterized distilled water. Reproduction in both media was again by division cysts and through six generations. When rupturing began to occur at this point, a third transfer of ciliates to their respective media was made. In the third transfer the organisms in conditioned medium reproduced for three generations by division cysts, one generation by atypical division cysts and two generations by fission division. In the distilled water reproduction was by division cysts. Four more transfers were carried out. In the conditioned medium there was fission division only for five or six generations when the usual division cysts appeared, followed by resting cysts. In the distilled water only division cysts were produced. It will be noted that all ciliates in these experiments came from the original stock strain, yet a drastic modification of their environment rendered them nonsusceptible to the action of the conditioned medium until after the fourteenth generation.

Ciliates from the August 12 *M* series reacted quite differently. In bacterized conditioned medium reproduction was by fission division only for six generations, followed by

division cysts and resting cysts. In bacterized distilled water, however, five fission-division forms were observed in the seventh generation. Seven consecutive transfers were made to bacterized distilled water. Fission division steadily increased until on the seventh transfer it was 100 per cent. All the ciliates on the two (duplicate) cover glasses were then transferred to a tube with 6 ml. of bacterized distilled water. This tube was re-bacterized every 48 hours. Reproduction, as seen microscopically in loops of the tube's contents, continued by fission division. At 2-day intervals 0.5 ml. quantities of the liquid were centrifuged at high speed and tested for the conditioning factor. Each 0.5 ml. was replaced by 0.5 ml. of distilled water. The conditioning factor first appeared in 16 days (produced some fission division in the fifth generation of S 76) and in 20 days was sufficiently concentrated to produce 100 per cent fission division (normal concentration) for six generations of S 78. A loop from this tube was inoculated into a fresh tube of bacterized distilled water and was treated in the same manner. The conditioning factor in normal concentration was demonstrated in 9 days. The experiment was continued until, in a fresh transplant, the conditioning factor was produced in sufficient quantity in 24 hours to induce 100 per cent fission division in the third or fourth generation of the stock strain. In 48 hours, however, the conditioning factor was present in normal concentration.

Nature of the conditioning factor.—The concentrations of the conditioning factor in the experimental tube on July 28 and on August 28 was determined with ciliates from S 55 and S 68 respectively. Ciliates were placed in bacterized concentrated (1:1), and bacterized dilutions (distilled water) of conditioned medium (1:2.5, 1:5, 1:10, 1:50, and 1:100). In the concentrated and the 1:2.5 dilution, reproduction was by fission division to the sixth generation; in the 1:5 dilution, to the third or fourth generation; no fission-division forms were found in the 1:10 or higher dilutions. At one time some of the *M* ciliates produced conditioned medium of sufficient concentration to be effective in a dilution of 1:10, but not 1:25. This was the strongest conditioned medium obtained.

When relatively large amounts of conditioned medium were made available by the *M* ciliates, 1 ml. was evaporated to dryness at atmospheric pressure, 1 ml. *in vacuo* over sulphuric acid, and each taken up in 0.2 ml. of distilled water. Both preparations were effective in producing fission division in normal ciliates, but approximately half of the potency of the conditioned medium had been lost in drying. Five milliliters of the conditioned medium was reduced to 0.5 ml. *in vacuo*. The concentrated material was capable of causing fission division, but again was about one-half as potent as the unconcentrated substance. When the concentrated material was diluted ten times, it produced no effect on the ciliates. An actual destruction or alteration of the conditioning factor seems to occur both in drying and in concentrating.

The potency of the conditioned medium was impaired by heating to 50° C. for 5 minutes and completely destroyed or inactivated at the same temperature for 10 minutes.

All attempts to precipitate the conditioning factor by alcohol failed. Berkfeld filtrates of conditioned medium lacked the conditioning factor. When powdered Keeselgohr was added to the conditioned medium, then removed by high-speed centrifugation, the supernatant fluid was also devoid of the active principle. This would seem to indicate that the conditioning factor was not removed by filtration per se, but by adsorption on the electronegative particles of which the Berkfeld filter is composed.

On July 28 a small amount of conditioned medium was set aside at room temperature in a sealed ampule. Weekly, for 7 weeks, the ampule was opened, tested for the active

principle, and resealed. For 4 weeks the activity of the conditioned medium was unimpaired, a slight decrease was noted by the fifth week, and a marked decrease by the sixth week. By the seventh week the conditioning factor had completely disappeared. Conditioned medium stored in a rubber-stoppered tube at 2° C. depreciated only slightly in 2 months.

Confirmatory production of conditioned medium by the method first described.—On June 30 stock ciliates (S 42) were placed in 6 ml. of bacterized distilled water in four tubes. These tubes were bacterized at weekly intervals, each tube receiving the growth on a 24-hour agar slant culture. At the time these tubes were set up, single ciliates from S 42 were placed in four hanging drops of bacterized distilled water. After the sixth generation the ciliates from each drop were transferred to each of four tubes containing 5 ml. of bacterized distilled water. Thirty-six hours later the total contents of each of these last tubes were transferred to each of four bottles containing 45 ml. of bacterized distilled water. This procedure is necessary since single ciliates do not ordinarily survive in large volumes of medium (Kidder and Stuart, 1939*b*). The bottles, in contrast to the tubes, had to be bacterized at 2- or 3-day intervals with approximately one-half of a slant culture to insure survival of the ciliates.

Three of the four tubes and one of the four bottles showed the presence of the conditioning factor in weak concentration on October 4, producing a few fission divisions in the fourth, fifth, or sixth generation of stock ciliates (S 85). On November 16, the conditioned medium from three of the tubes produced 100 per cent fission division of S 104 ciliates. The conditioning factor was present in the other tube and in all the bottles but in lower concentrations.

DISCUSSION

It is well known that marked changes are sometimes produced in the medium in which organisms are growing. In some cases the changes are detrimental to the subsequent growth of the same or other species, as evidenced by lowered division and increased death-rates. In other cases the conditioned medium seems to increase the division-rate and lower the death-rate. Changes in the oxidation and reduction potential, in the hydrogen-ion concentration, in the food supply, in the concentration of metabolic products, and many other factors, doubtless play a part in altering the medium. In the present work none of these factors has been studied. In fact it is not known whether the ciliates or the bacteria are responsible for the effects noted in our experiments. There is, however some evidence which tends to show that the ciliates, more than the bacteria, condition the medium. In the original experimental tube approximately 5 months elapsed before the ciliates reproduced by fission division and before the medium was conditioned to the point where it would cause normal stock ciliates to reproduce by fission division. Strain 3, from the wild, reproduced by fission division after several transplants to bacterized distilled water. Unfortunately, no attempt was made to condition medium with this strain. A strain of stock ciliates which had been reared in a pure culture of *Mycobacterium* produced fission-division forms in a single transplant to bacterized distilled water and was later able to condition fresh bacterized distilled water in 24 hours so that the conditioned medium caused normal stock ciliates to reproduce by fission division. Since at the time fission division occurred in these three strains of ciliates the bacterial flora was very similar, it is possible that the previous environment of the ciliates had more influence than

the bacteria in conditioning the medium. Furthermore, the effect of the conditioned medium appears to be species specific.

It is possible that the conditioning factor arises spontaneously in the ciliate as might an adaptive endo-enzyme in response to one or more environmental factors. Under this condition only the ciliate in question and its progeny are affected. If the environment continues to be suitable for the production of the conditioning factor, it may be liberated into the medium, eventually reacting on all ciliates of the same species.

From the preceding evidence it is obvious that the method of reproduction in *Colpoda* is subject to alterations according to certain changes in the environment of the ciliate. A single *Colpoda* destined to reproduce by a division cyst can be made to undergo fission division in conditioned medium. Other types of reproduction have been encountered, such as the atypical division cyst. In this type there appears to be premature release of the ciliates some time after the cilia form, due possibly to a weak cyst wall. In fission-division cysts the entire cyst wall ruptures before cilia have formed. In fission division no cyst wall is formed at all. It might seem that the conditioned medium merely exerts a lytic action on the wall of the division cyst. If such action does take place, it cannot account for all the reactions of these ciliates in conditioned medium. In strong conditioned medium ciliates produce one generation of regular division cysts before going into resting cysts. This occurs even when more conditioned medium is added a generation or two before the division cysts are to be formed. In bacterized conditioned medium 6 generations are usually produced by fission division before division cysts occur, while in unbacterized conditioned medium division cysts followed by resting cysts occur after the second generation in the same concentration of conditioned medium. Ciliates placed in conditioned medium about one hour before reproducing form perfectly normal division cysts. Ciliates placed in conditioned medium for 1 to 2 hours immediately after normal division and then washed free of conditioned medium, reproduce by fission division. Furthermore, as already pointed out, ciliates from *M* series reproduced in bacterized distilled water for many generations by fission division before the conditioning factor was demonstrable in the medium in which the ciliates were growing. It is difficult to escape the conclusion that the conditioning factor acts on some internal factor or mechanism in the ciliates causing a change in their method of reproduction.

As previously pointed out, this strain of *Colpoda* has been observed to reproduce in several different ways. Moreover, when the conditions are right, the various types of reproduction do not occur haphazardly but in orderly sequence. In the proper concentration of conditioned medium a ciliate has been observed to reproduce by a regular division cyst. The next generation reproduced by atypical division cysts, in which the cyst wall seems weak. The next generation reproduced by fission-division cysts, in which the cyst wall ruptures as soon as the second division within the cyst is completed. This was followed by a fission division, in which no cyst wall is formed and in which there is some question as to whether the cilia are completely lost during the division. In fission division numerous cases have been observed wherein a second division did not occur and the two individuals developed quite normally. Finally, eighteen cases have been carefully followed in which the ciliates reproduced by simple fission, while continuing to swim about in the medium. The evolutionary implication of this train of events is clear, but for the present must remain only an implication.

CONCLUSIONS

1. *Colpoda* may reproduce in a variety of ways, and under certain conditions the type of reproduction can be experimentally controlled.

2. It has been demonstrated that conditioned medium can be used to induce different types of reproduction in this strain of *Colpoda*.

3. The conditioning factor is unstable, thermolabile, cannot be concentrated, and is adsorbed on a Berkfeld filter. It appears to be species specific.

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THE VITAMIN B COMPLEX AND THE GROWTH OF COLPIDIUM STRIATUM[1]

(Five figures)

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EARLIER attempts to determine the limiting factors for growth of *Colpidium striatum* in bacteria-free cultures indicated that small quantities of some substance or substances found in yeast extract, partially hydrolized casein, and liver infusion were responsible for the success or failure of a medium to support continued growth of this ciliate (Hall and Elliott, 1935). Traces of these substances were essential for continued growth of this protozoan in such incomplete proteins as zein, gliadin, and gelatin, even though the missing amino acids were replaced. This fact led to the inevitable conclusion that some factor or factors of the vitamin B complex were involved. Therefore, "pantothenic acid," a universally occurring substance similar to vitamin B_2 (Williams *et al.*, 1933) was investigated and found to have an accelerating effect on the growth-rate of this protozoan (Elliott, 1935). Since pantothenic acid has not been identified as a specific vitamin, this investigation was planned to study the role played by certain purified members of the vitamin B complex in the nutrition of *C. striatum*. The factors investigated were thiamin chloride, riboflavin, and a vitamin B_6 concentrate.

MATERIAL AND METHODS

The experimental organism employed in this investigation was *C. striatum*, a ciliate protozoan isolated by the author in pure-line, bacteria-free cultures several years ago (Elliott, 1933). This organism is definitely mammal like in its food requirements, since it thrives only on complex nitrogen and carbon sources (Elliott, 1935*a*, 1935*b*). For experimental purposes the protozoa were cultured in 250 cc. pyrex flasks, and frequent bacteriological tests assured complete freedom from contamination with other microorganisms. The basic culture medium used throughout the studies, with the exception of Series I, was composed of the following substances:

$NaCl$	0.003 gm.	$FeSO_4$	Trace
KNO_3	0.002 gm.	Tryptone	20.0 gm.
$CaSO_4$	0.020 gm.	Dextrose	2.0 gm.
$MgSO_4$	0.005 gm.	Glass-distilled H_2O	1.0 liter

The solution exclusive of the tryptone and dextrose is spoken of as "artificial tap water." For experimental purposes large quantities of the basic medium were prepared in pyrex flasks, one-half of which were rendered vitamin B-free in order to determine the exact effect of the vitamin tested. Heretofore, research work on the nutrition of protozoa usually involved a study of the accelerating effect of the substance in question on the

[1] This investigation was supported in part by grants from the Minnesota Academy of Science and the American Association for the Advancement of Science.

growth-rate of the organism in a medium already adequately provided with food requirements. This method failed to indicate whether or not the tested substance was essential in the nutrition of the experimental organism. In the present investigation the vitamin tested was removed from the medium and subsequently replaced by the purified product which indicated whether or not it was an essential growth factor for *Colpidium*. The vitamin B complex was destroyed in the basic medium by adjusting the pH to 9.6 and then heating in the autoclave for one hour at 20 pounds pressure. The pH was then readjusted to 5.8–6.2 before final sterilization. Experiments showed that with this method growth in the treated basic medium was negligible in the first transfer and ceased altogether in the second, indicating that most of the vitamin B complex had been destroyed. Tests for vitamin B_1 with 2.6-dibromoquinone-chloroimide according to the method of Raybin (1938) were negative. Vitamin-free casein was also employed as an alternative method but was unsatisfactory for two reasons: (1) It was necessary to digest the casein with pepsin before the ciliates were able to utilize it, and this involved added complications. (2) It was discovered by several experiments that thiamin was present in sufficient quantities to permit continued growth which impaired the validity of the results. Therefore, the heat treatment was relied upon throughout most of the experiments.

Once the basic medium was rendered vitamin B-free and the pH adjusted to approximately 6.0 which is optimum for *Colpidium*, it was dispensed to 250-cc. flasks in 100 cc. quantities. The vitamin was added to the medium which was again autoclaved, this time for sterilization only which required 15 pounds for 15 minutes. The flasks were then inoculated with the ciliates which had previously been thoroughly washed according to the following procedure. A rapidly growing culture in the untreated basic medium was centrifugalized and the supernatant fluid decanted off; this process was repeated several times with artificial tap water as the diluent. The protozoa were then added to a 250-cc. flask containing 100 cc. of sterile artificial tap water. From this inoculating flask the organisms were transferred by means of graduated pipettes in measured amounts to the test flasks. The inoculation flask was always thoroughly shaken before each transfer in order to insure equal numbers of ciliates in each inoculum. The entire process involved aseptic technique, and frequent bacteriological tests indicated that contaminations were rare; all contaminated cultures were eliminated from the experiments. By this procedure a negligible amount of the original stock culture medium was transferred to the test flasks, which assured reasonably accurate results on the first series of transfers; however, several successive transfers were always made in order to confirm the effect observed in the initial one. The test flasks were incubated in a constant temperature bath at $26° \pm 0.02°$ C. The amount of growth was determined by means of the protozo-ocrit method (Elliott, 1939). The initial inoculations were estimated and found to be uniform in all cases. Estimations of growth were made at regular intervals, usually 12 or 24 hours, throughout the growth period of the cultures. It was found quite satisfactory to make estimations on living samples which consisted of 10 cc. of the culture withdrawn aseptically from the test flasks. Duplicate and triplicate cultures were always maintained, and where differences varied more than 5 per cent the series was repeated in its entirety.

The thiamin chloride used in this investigation was obtained in 0.01-gm. ampoules from Merck and Company; the vitamin-free casein, crystalline riboflavin, and vitamin B_6 concentrate from the S.M.A. Corporation; the tryptone, isoeletric casein, and gelatin from the Difco Laboratories; and the 2, 6-dibromoquinone-chloroimide from the East-

man Kodak Company. The vitamins were prepared in solution shortly before use in the tests to insure maximum activity. The initial and final pH determinations were made colorimetrically.

THIAMIN CHLORIDE

Series I.—In view of previous efforts to culture *C. striatum* on incomplete proteins, it was thought worth while in this initial series to attempt to grow this organism on one complete protein (isoeletric casein), one incomplete protein (gelatin), and one partially hydrolized protein (tryptone, an enzymatic casein digest), supplementing each with thiamin chloride. Half of the basic medium was adjusted to pH 8.8 and treated with heat in the autoclave for 30 minutes at 15 pounds pressure. This heat treatment was later shown to be inadequate to destroy all the vitamin B_1. The pH was then adjusted to 6.0 in the treated media and distributed to six flasks for each protein; a similar set of flasks contained the control media which were identical with the test media except that during the heat treatment the pH was retained at 6.0. To one-half of the flasks of both the control and the test media was added thiamin chloride making a final concentration of 1:500,000. The flasks were then autoclaved for sterilization and inoculated with approximately equal numbers of washed ciliates. They were then incubated in the constant-temperature bath for 6 days. The volume of protozoa was then estimated for each flask by means of the protozo-ocrit.

It was obvious from the results of this series (Fig. 1) that none of the flasks containing gelatin and casein supported growth to any appreciable extent; furthermore, there was no evidence of any effect of the added thiamin. The heating at a high pH seemed to have rendered the gelatin even less suitable for growth than the untreated product. Transfers to a second series of flasks resulted in no growth at all. It is apparent that thiamin chloride is not the limiting factor for growth when gelatin and casein are employed as basic proteins. However, the results with tryptone, a partially hydrolized casein product, are quite different. The heat treatment seemed to have had little effect in destroying the vitamin B_1, since growth is nearly as good in the untreated medium. The supplemented thiamin brought about accelerated growth to a significant degree in the treated culture. On the other hand, the untreated tryptone supported equal growth with and without the addition of thiamin—a fact which indicates that there was sufficient thiamin in the original tryptone to support excellent growth. However, it is not clear why growth in the treated tryptone plus vltamin B_1 was better than in the untreated tryptone supplemented with thiamin.

The results of this series indicate that thiamin chloride is not the limiting growth factor in cultures employing whole casein or gelatin as a source of nitrogen, although partially hydrolized casein responds to the vitamin, providing heat treatment has occurred.

Series II.—In this series the heat treatment was made more drastic by extending the time of autoclaving to one hour and raising the pressure to 20 lbs. and the pH to 9.6. Twelve flasks were prepared, six of which were treated to remove the vitamin B complex; the other six were autoclaved along with the first, the only difference being that the pH was set at 6.0 instead of 9.6. Thiamin chloride was added to one-half of the treated and to one-half of the untreated flasks in a concentration of 1:100,000. The remaining flasks were retained as controls. Inoculation and incubation were performed as in Series I, and estimations of growth were made at 12-hour intervals. The growth record (Fig. 2) indicates two distinct facts: (1) The vitamin was almost completely destroyed in the

treated medium by the heating process at pH 9.6. (2) The supplemented thiamin was definitely the limiting growth factor. This last fact was indicated since growth was almost as good in the treated tryptone plus thiamin as it was in the untreated protein. Addition of vitamin B_1 to the untreated tryptone had no effect whatever, indicating that this protein possesses sufficient thiamin naturally to support maximum growth of *C. striatum*.

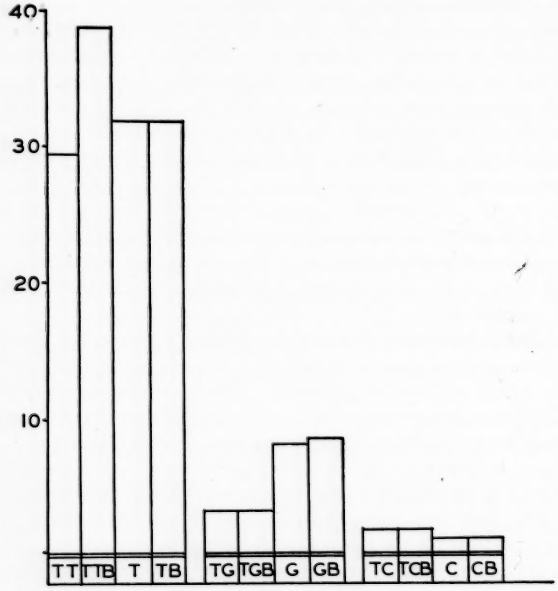

FIG. 1.—Series I. Volumes of packed ciliates in cubic millimeters plotted for the various proteins which are indicated as follows: *TT* = heat-treated tryptone; *TTB* = heat-treated tryptone plus thiamin; *T* = untreated tryptone; *TB* = untreated tryptone plus thiamin; *TG* = heat-treated gelatin; *TGB* = heat-treated gelatin plus thiamin; *G* = untreated gelatin; *GB* = untreated gelatin plus thiamin; *TC* = heat-treated casein; *TCB* = heat-treated casein plus thiamin; *C* = untreated casein; *CB* = untreated casein plus thiamin. There is a statistical-significant difference between *TT* and *TTB;* not between *TTB*, *T*, and *TB*.

In order to prove that vitamin B_1 is absolutely essential for growth of this ciliate, it was necessary to continue the cultures through several transfers. This was done, and it was demonstrated that growth after the eighth transfer was equally as abundant as in the first. Therefore, it is concluded that thiamin chloride is essential in the nutrition of *C. striatum*.

EFFECT OF DIFFERENT CONCENTRATIONS OF THIAMIN CHLORIDE

Since it was clear that the limiting factor for growth in the previous series was thiamin, it was necessary to determine which concentrations supported optimum growth and whether or not excessive amounts were injurious.

Series III.—In this series 18 flasks were prepared with heat-treated basic medium, 100 cc. to each flask. The thiamin chloride was added to the flasks in the following concentrations: 1:10,000; 1:100,000; 1:1,000,000; 1:10,000,000; 1:1,000,000,000. The flasks were run in triplicate with three controls containing no vitamin. Inoculations and incubation were carried out as usual, and samples were taken at 24-hour intervals after the first day of growth; two samples were taken during the fourth day of incubation. The

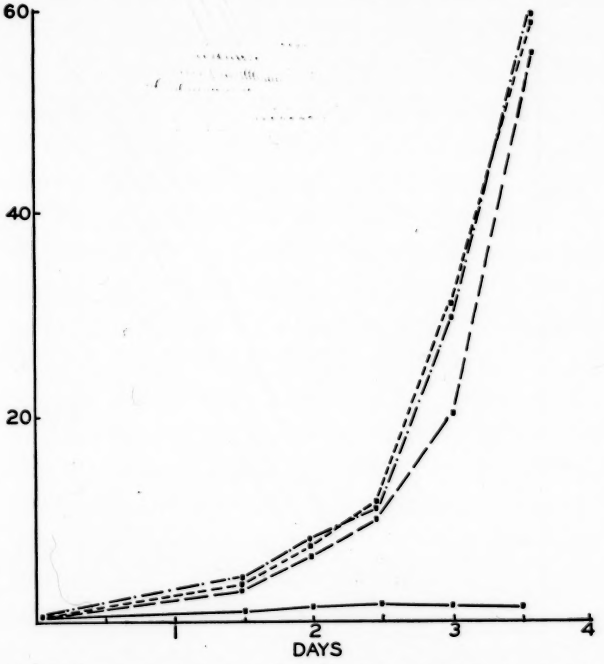

FIG. 2.—Series II. Volumes of packed ciliates in cubic millimeters plotted against time in days. The various media are indicated as follows: —— heat-treated tryptone; — — — heat-treated tryptone plus thiamin; - - - - - - untreated tryptone; — • — • — • untreated tryptone plus thiamin.

record (Fig. 3) indicates the same sort of effect observed in Series II. It is clear that a vitamin B_1 concentration of 1:1,000,000,000 was insufficient to support optimum growth. Concentrations above this seemed adequate at the 84-hour period of growth, but concentrations of 1:10,000 produced the best results. When growth continued another 12 hours, the flasks with concentrations of 1:100,000 and 1:10,000 fell off in their growth-rate, while those with concentrations of 1:1,000,000 and 1:10,000,000 continued to support superior growth. Additional experiments, not recorded here in detail, with concentrations as high as 1:2,000 of thiamin showed no better growth than those with 1:10,000,000; on the other hand, no injurious effects were noted with such high concentrations. It is concluded, therefore, that concentrations of thiamin chloride ranging from 1:10,000 to 1:10,000,000 are sufficient to support excellent growth.

RIBOFLAVIN

Even though thiamin chloride seemed to be the limiting factor for growth in the basic medium of the previous series, it was thought worth while to determine whether or not crystalline riboflavin—vitamin B_2—might be able to replace thiamin in the nutrition of *C. striatum*. For that reason Series IV was conducted.

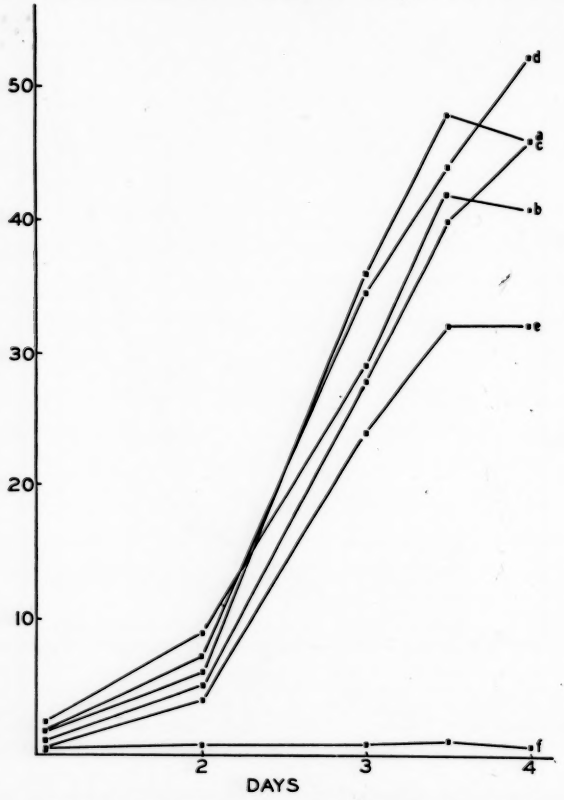

FIG. 3.—Series III. Volumes of packed ciliates in cubic millimeters plotted against time in days. The concentrations of thiamin are indicated as follows: $a = 1:10{,}000$; $b = 1:100{,}000$; $c = 1:1{,}000{,}000$; $d = 1:10{,}000{,}000$; $e = 1:1{,}000{,}000{,}000$; f = control with no thiamin.

Series IV.—Sixteen flasks of heat-treated basic medium were prepared, each containing 100 cc. of medium. Crystalline vitamin B_2 was added to these flasks in the following concentrations: 1:2,500; 1:25,000; 1:250,000; 1:2,500,000; 1:25,000,000; 1:1,000,000,000. The series were run in duplicate. Two flasks containing no riboflavin and two containing thiamin chloride in concentrations of 1:100,000 were maintained for comparative

purposes. Sterilization, inoculation, and incubation were conducted as usual. Estimations of growth were made at 24-hour intervals after the second day of growth. The record (Fig. 4) demonstrates that growth was maintained at a low level for the first five days in flasks containing concentrations of 1:2,500, 1:25,000, 1:250,000,and 1:2,500,000; growth was no better than the control in the two most dilute flasks, so no record was

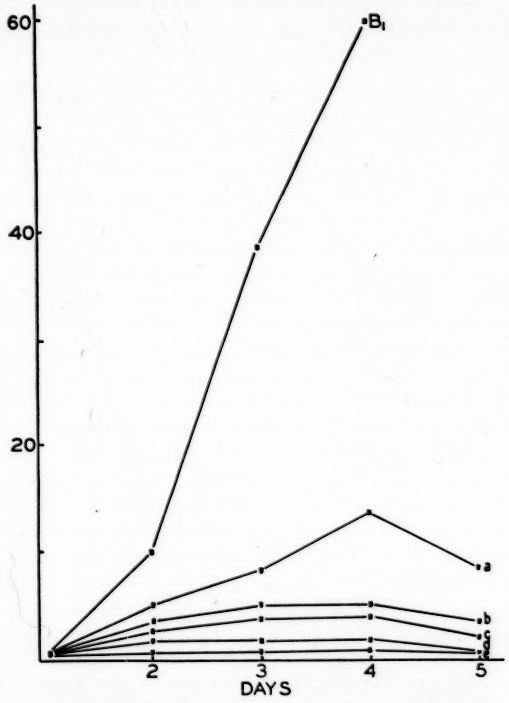

FIG. 4.—Series IV. Volumes of packed ciliates plotted against time in days. The concentrations of riboflavin are indicated as follows: $a = 1:2,500$; $b = 1:25,000$; $c = 1:250,000$; $d = 1:2,500,000$; e = control with no riboflavin; B_1 = thiamin.

made. The optimum growth in the flasks containing thiamin was reached on the fourth day. While growth was supported at a very low level with riboflavin at certain concentrations, it was clear that this might not continue through several successive transfers. In order to check this point transfers were made, and it was found that growth was just perceptible in the second transfer and ceased altogether in the third. Parallel tests with digested vitamin-free casein indicated similar results. Cultures containing thiamin in addition to riboflavin gave no better growth than those with vitamin B_1 alone. It was concluded, then, that riboflavin is incapable of replacing thiamin chloride in the nutrition of *C. striatum*.

VITAMIN B_6

Even though riboflavin appeared to be unnecessary in the nutrition of *Colpidium*, it is possible that certain other members of the vitamin B complex might possess the properties necessary to replace vitamin B_1. For that reason a concentrate containing vitamin B_6 was investigated.

Series V.—The procedure in this case was similar to that employed in Series IV. Fourteen flasks of treated basic medium were prepared, to which the following concentrations

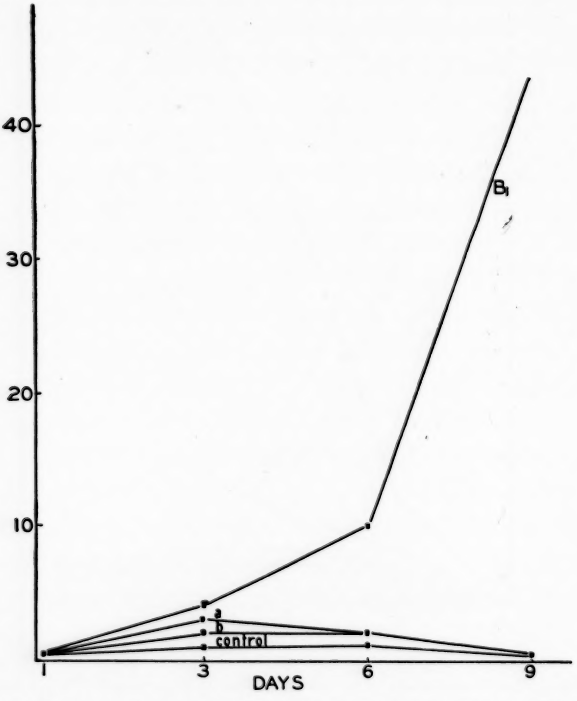

FIG. 5.—Series V. Volumes of packed ciliates plotted against time in days. The concentrations of vitamin B_6 are indicated as follows: a = 0.1 cc.; b = 2.5 cc.; *control* = no vitamin B_1; B_6 = thiamin.

of vitamin B_6 were added: 0.1 cc. to the first pair of flasks, 0.5 cc. to the second, 2.5 cc. to the third, 5 cc. to the fourth, and 15 cc. to the fifth. Flasks receiving smaller quantities than 15 cc. were supplemented with proper additions of artificial tap water. Two controls were run, as well as two flasks containing thiamin (1:100,000) for comparative purposes. Sterilization, inoculation, and incubation were conducted as in previous series. Samples were taken and estimated on the third, sixth, and ninth days of incubation. The record (Fig. 5) indicates that growth, as compared with the controls, was slightly stimulated in flasks containing concentrations of 0.1 cc. and 2.5 cc. Concentrations higher than these inhibited growth altogether and therefore were not recorded. For compari-

son, growth in the flasks containing thiamin demonstrated a long-lag phase but were normal otherwise. Transfers to a second set of flasks demonstrated complete cessation of growth at all concentrations. Tests with basic medium supplemented with thiamin and riboflavin as well as vitamin B_6 concentrate, showed no better growth than those with thiamin alone. It is concluded then that the factors existing in the vitamin B_6 concentrate employed in this investigation were unable to replace B_1 in the nutrition of *C. striatum*.

DISCUSSION

The fact that certain substances, now recognized as probable members of the vitamin B complex, influence the growth of micro-organisms has been a well-known fact for many years. Wildiers (1901) obtained his so-called "bios" from yeast which had a marked effect on the growth-rate of the same organism from which it was derived. Later, Williams *et al.* (1933) isolated a universally occurring substance, "pantothenic acid," which had a pronounced effect on the growth of yeast and more recently on *C. striatum* (Elliott, 1935) and was thought to be closely related to vitamin B_2. Crystalline thiamin chloride (vitamin B_1) has recently been shown to be essential in the nutrition of many plants and animals. Even such simple forms as propionic acid bacteria require this substance or certain of its decomposition products (Tatum, Wood, and Peterson, 1936). It is also demanded by *Staphylococcus aureus* (Knight, 1937). Isolated pea and tomato roots require thiamin in their normal growth (Bonner, 1937; Robbins and Bartley, 1937). It is essential in normal insect development (Trager and Subbarow, 1938), and its role in mammalian nutrition is well known. Vitamin B_1 apparently plays an important role in the normal nutrition of all plants and animals so far investigated.

Because of its universal need the writer expected that a similar demand would be found among the protozoa. Hall and Elliott (1935) suspected that certain members of the vitamin B complex might be the limiting factor or factors for growth of the ciliate, *C. striatum;* this thought was further suggested when it was learned that pantothenic acid caused a marked acceleration of growth (Elliott, 1935). Later it was demonstrated and reported in abstract form (Elliott, 1937) that crystalline vitamin B_1 played an important part in the growth of this ciliate. Likewise, Lwoff and Lwoff (1937), working with a closely related ciliate, *Glaucoma piriformis*, and Lwoff, M. (1937), with a flagellate, *Strigomonas oncopelti*, showed a similar effect. However, certain protozoa like *Polytoma obtusum* require neither part of the thiamin molecule (Lwoff and Dusi, 1937). Both *Glaucoma* and *Strigomonas* require the intact molecule which is also true of certain plants as well as of mammals. While the pyrimidine and thiazole portion of the thiamin molecule were not investigated in this series of experiments, it is highly probable that the requirements of *Colpidium* would be little different from those of *Glaucoma*.

The fact that thiamin was not the limiting factor in cultures containing gelatin (Series I) was not surprising in view of the recent report by Hall (1938), showing that pimelic acid made possible the successful culture of *C. campylum* in a gelatin medium containing dextrose. This fact does not preclude thiamin as an essential growth factor for *C. campylum*, since no effort was made to control this phase of the experiment; it merely demonstrates that pimelic acid is necessary for continued growth of this ciliate in gelatin.

Earlier experiments (Elliott, 1933) demonstrated clearly that *C. striatum* elaborates enzymes capable of coagulating casein in litmus milk, but it has never been possible to culture this ciliate on pure unhydrolized casein, although it has been grown for several

years in the casein enzymatic digest, tryptone. It would appear that the ciliate is incapable of producing enzymes which will hydrolize this protein. The supplemented thiamin in Series I made no difference in the matter of utilization of casein by the ciliate.

Vitamin B_2 (lactoflavin or riboflavin) is an essential factor in the diet of mammals (Ansbacher *et al.*, 1936), as well as of other animals. For instance, Trager and Subarrow (1938) have shown that not only thiamin but also riboflavin is essential for the normal development of the mosquito larva, *Aedes aegypti*. The role played by this vitamin among the protozoa is unknown. It was obvious from the results of this investigation (Series IV) that riboflavin could not replace thiamin in the nutrition of *C. striatum*. This was an anticipated result, because all the vitamin B complex was supposed to have been destroyed with the heat treatment, and the supplemented thiamin was adequate to support abundant growth through eight successive transfers. It is possible that the heat treatment failed to destroy all the vitamin B_2, and a sufficient amount remained to permit excellent growth such as occurred in Series I, II, and III where thiamin was the only added vitamin. However, parallel experiments with so-called vitamin-free casein revealed similar results. Here, again, perhaps all the lactoflavin was not removed from the casein. Many commercial brands of vitamin-free casein have been shown to contain traces of lactoflavin (chemically identical to riboflavin) (Supplee *et al.*, 1936). It is interesting that this ciliate which is so mammal like in all other food requirements can subsist without vitamin B_2. The present series of experiments in no way prove this point; they merely show that riboflavin cannot replace thiamin. Other more detailed experiments are being conducted at present to discover, if possible, the exact role of riboflavin in the nutrition of *C. striatum*.

There is nothing known about the effect of vitamin B_6 on the growth of protozoa. The present investigation shows clearly that this vitamin will not replace thiamin in the nutrition of the ciliate studied. Hutner (1936) has shown that *Euglena gracilis* requires "a water soluble, thermostable material widely distributed in nature and present in relatively high concentrations in autolyzed yeast and urine." According to a series of experiments not reported here, this factor is not thiamin; it may be riboflavin or vitamin B_6. It is possible that not all the vitamin B_6 was destroyed in the heat treatment and therefore remained in small quantities in the basic medium; then when vitamin B_1 was omitted from the medium in Series V, growth was impossible. However, growth in Series I, II, and III would be very good because sufficient vitamin B_6 would have remained in the basic medium to make this possible. Whether or not this is true must await further study.

SUMMARY

Three purified members of the vitamin B complex were studied in respect to the nutrition of the ciliate protozoan, *C. striatum*, in bacteria-free cultures. Crystalline thiamin chloride (vitamin B_1) was proved to be a limiting factor for growth in media free from this substance. Heating a casein digest, tryptone, in the autoclave for one hour at 20 lb. pressure and pH 9.6 destroyed most of the thiamin. Vitamin B_1 was most effective in concentrations ranging from 1:10,000 to 1:10,000,000. Crystalline riboflavin and vitamin B_6 (concentrate) could not supplant thiamin in the nutrition of this ciliate.

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RATE OF GROWTH OF DAPHNIA LONGISPINA AS AFFECTED BY FISH-CONDITIONED WATER AND BY CERTAIN FISH EXTRACTS[1]

(One figure)

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RESULTS of studies by Allee and associates have shown that, other things being equal and under the conditions of their experiments, isolated goldfish grew more rapidly if placed in water in which other goldfishes had lived than if in uncontaminated but otherwise similar water. Further investigations bear out the foregoing statement and extend the work to include assays of a protein extract of the surface of goldfishes (Allee, Oesting, and Hoskins, 1936).

The investigations reported here were the result of a bringing-together of the senior author's interest in the physiology of *Daphnia* with this work on the growth-promoting qualities of fish-conditioned waters and of extracts of fish origin. This afforded an opportunity to test the value of *Daphnia* as an assay organism for these growth-promoting substances and to ascertain the effect of heterotypic conditioning on the growth of *Daphnia*.

Kawajiri (1928) concluded from studies on population density of rainbow trout that the survival rate increased and the rate of growth decreased as the number of fry in a box increased. Adolph (1929) found that the maximum growth of amphibian larvae occurred with a single animal in the largest volume, although conditioned water could favor the growth of the individual. In 1932, Shaw working with the tropical fish *Platypoecilus maculata* and with *Amblystoma* tadpoles found that heterotypic conditioning favored growth more than did homotypic conditioning. Woodruff (1914) showed that the excretory products of *Paramecia* were toxic to themselves, as was also true of hypotrichs. The excretory products of hypotrichs, however, stimulated the reproductive rate of *Paramecia*.

Although many of the factors involved are not yet entirely clear, heterotypic conditioning apparently favors growth more than does homotypic conditioning, and hence assays of reputed growth-promoting materials are more readily made across than within species boundaries.

Results to be reported in this paper include tests of fish-conditioned waters, a crude extract of the skin of goldfishes, and a refinement of the crude extract designated as alkaline extract, for their effects on the growth of *Daphnia*.

Extracts were prepared by placing goldfish (*Carassius auratus*) into 0.025 N NaOH for one hour; the resulting fluid which resembles a clear syrup was strained through heavy cheesecloth, and the volume divided into two equal parts. One portion was used with-

[1] The senior author wishes to express his appreciation to the National Research Fellowship Board for the fellowship which made this research possible and to the University of Chicago, and both authors wish to thank Dr. W. C. Allee for his many helpful suggestions during the progress of the work.

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out further treatment and designated as "crude extract." The other portion was treated with 0.5 N H_2SO_4 until a heavy precipitate was formed. The precipitate, which was washed thoroughly and dried with absolute ethyl alcohol and ether, was weighed, and its weight was assumed to be the equivalent of the extracted portion remaining in the crude state. This dry precipitate dissolved in 0.01 N NaOH constituted the "alkaline extract." The solutions of both extracts were autoclaved for 15 minutes at 15 lbs. pressure and stored in an icebox. Extract controls consisted of NaOH in aqueous solution added to the culture media of *Daphnia* so that the alkalinity of the control would be approximately the same as that of the test.

Several types of fish-conditioned waters were used, namely, conditioned lake water (C.L.W.) from Lake Michigan, conditioned tap water (C.T.W.), charcoal filtered, and conditioned synthetic pond water (C.S.P.W.) (Allee, Oesting, and Hoskins, 1936). These conditioned waters were prepared for use in experiments, concurrently in progress, dealing with their effects on assay goldfish and were thus prepared anew daily.

Female *D. longispina* from Banta's Line 1284 were used as the experimental animals. They afford certain advantages for investigations herein reported. Data for critical comparisons may be obtained from a large number of animals, and many experimental groups with adequate controls may be included in a single series. A still more important advantage which *Daphnia* afford arises from the fact that they reproduce by diploid parthenogenesis and as members of a pure line (clone) exclude the probability of a differential influence of any heritable factors for growth.

The *Daphnia* were reared individually in 100 cc. of manure-infusion medium (Banta, 1921) maintained at 25° C. and contained in a 6-ounce widemouthed bottle. A small outside fish tank containing several goldfish served as the source of water for manure-infusion media of Series III. The source of water for Series IV, V, and VI, except normal control of Series VI, which utilized water from the outside fish tank, was tap water filtered through activated charcoal and gravel. The animals were measured (body length exclusive of the caudal spine) with the aid of an ocular micrometer at the time of their release from the mother's brood chamber, and each brood was then distributed evenly among the various experimental and control groups. They were measured at the beginning of each instar up to and including the sixth and were transferred to fresh medium after each measurement. *Daphnia* do not increase in size except immediately following ecdysis, hence each measurement represents the summation of growth during any given instar.

The dilution for the extracts was 1 part fish extract to 400,000 parts culture medium. Media treated with fish-conditioned water were prepared by adding 20 cc. of conditioned water of known strength to 80 cc. of regular manure-infusion medium so that the coefficient of conditioning (Allee, Oesting, and Hoskins, 1936) was 25, except for one case in Series III where a set with a coefficient of 10 was also prepared. Control bottles, similarly, had 20 cc. of untreated lake, pond, or filtered tap water added to the 80 cc. of regular medium.

Table 1 presents a composite view of the results obtained. In Series III, the averaged first growth increment for the experimental groups was significantly[3] larger than their controls, as is shown in the column of statistical probabilities.

[3] Statistical significance was calculated in terms of P and is based on "Student's" method for finding statistical significance of relatively small numbers of paired observations. P = 0.05 is equivalent to three times the probable error; this valus is taken as the extreme upper limit of statistical significance.

TABLE 1

INCREASE IN BODY LENGTH BY INCREMENTS OF *D. longispina* AS AFFECTED BY VARIOUS SUBSTANCES OF FISH ORIGIN

Treatment*	No. of Animals	Birth Length (Mm.)	First Increment (Mm.)	Statistical Probabilities	Second Increment	Statistical Probabilities	Third Increment	Statistical Probabilities	Fourth Increment	Statistical Probabilities	Total Body Length (Mm.)	Statistical Probabilities	Fifth Increment	Statistical Probabilities	Total Body Length (Mm.)	Statistical Probabilities
Series III:																
Norm. cont.	13	.714	.179		.269		.237		.227		1.626		.199		1.825	
Cond. l.w. (10)	14	.711	.210	0.000	.252	0.424	.303	0.007	.219	0.150	1.695	0.073	.214	0.381	1.909	0.001
Cont. l.w. (10)	14	.713	.179		.264		.252		.252		1.660		.198		1.858	
Cond. l.w.	13	.707	.211	0.016	.237	0.001	.294	0.002	.223	0.493	1.672	0.435	.265	0.052	1.937	0.030
Cont. l.w.	13	.719	.183		.281		.247		.238		1.668		.198		1.866	
Alk. ext. I.	12	.716	.211	0.000	.258	0.368	.299	0.001	.225	0.212	1.709	0.008	.236	0.035	1.945	0.000
Alk. ext. cont.	12	.706	.185	0.000	.273	0.424	.266	0.000	.207	0.333	1.637	0.000	.172	0.001	1.809	0.000
Alk. ext. II.	13	.713	.204		.260		.325		.226		1.728		.260		1.988	
Crude ext. I.	13	.710	.210	0.000	.270	0.368	.319	0.000	.250	0.162	1.759	0.001	.236	0.018	1.995	0.000
Crude ext. cont. I.	13	.725	.180		.256		.243		.220		1.624		.186		1.810	
Crude ext. II.	13	.717	.214	0.000	.267	0.046	.304	0.000	.254	0.162	1.756	0.000	.248	0.027	2.004	0.000
Crude ext. cont. II.	12	.713	.179		.294		.235		.221		1.642		.181		1.823	
Series IV:																
Norm. cont.	20	.650	.172		.293		.277		.319		1.711		.053		1.764	
Cond. filt. t.w.	20	.650	.204	0.000	.263	0.000	.335	0.000	.213	0.000	1.665	0.549	.125	0.000	1.790	0.028
Cont. filt. t.w.	20	.650	.173		.297		.276		.258		1.681		.065		1.746	
Alk. ext. I.	20	.649	.207	0.000	.270	0.000	.344	0.000	.253	0.000	1.723	0.230	.136	0.000	1.859	0.000
Alk. ext. cont.	20	.647	.172	0.000	.297	0.000	.283	0.000	.304	0.004	1.703	0.291	.050	0.000	1.753	0.000
Alk. ext. II.	20	.645	.202		.270		.358		.255		1.730		.141		1.871	
Cond. synth. p.w.	20	.643	.208	0.000	.264	0.000	.336	0.000	.196	0.001	1.647	0.549	.146	0.000	1.793	0.000
Cont. synth. p.w.	19	.644	.179		.283		.263		.265		1.034		.072		1.706	
Crude ext. I.	20	.650	.212	0.000	.277	0.000	.354	0.000	.255	0.000	1.748	0.842	.191	0.000	1.939	0.001
Crude ext. cont. I.	20	.651	.176		.297		.294		.325		1.743		.101		1.844	
Series V:																
Norm. cont.	15	.645	.176		.268		.247		.340		1.676		.295		1.971	
Cond. filt. t.w.	15	.645	.214	0.000	.253	0.000	.334	0.000	.333	0.089	1.779	0.000	.286	0.002	2.065	0.000
Cont. filt. t.w.	15	.651	.171		.286		.255		.351		1.714		.252		1.966	
Alk. ext. I.	15	.638	.210	0.000	.234	0.000	.283	0.009	.276	0.012	1.641	0.484	.290	0.484	1.931	0.089
Alk. ext. cont.	15	.635	.174	0.000	.271	0.000	.261	0.004	.320	0.317	1.661	0.549	.300	0.110	1.961	0.424
Alk. ext. II.	15	.644	.208		.236		.285		.303		1.676		.273		1.949	
Cond. synth. p.w.	15	.639	.214	0.000	.252	0.000	.356	0.000	.339	0.110	1.800	0.001	.297	0.001	2.097	0.000
Cont. synth. p.w.	15	.642	.173		.289		.250		.363		1.717		.252		1.969	
Crude ext. I.	15	.649	.210	0.000	.262	0.000	.341	0.000	.326	0.230	1.788	0.007	.308	0.004	2.096	0.000
Crude ext. cont. I.	15	.650	.172		.293		.256		.349		1.720		.264		1.984	
Series VI:																
Norm. cont. (f.t.w.)	20	.678	.188	0.484	.265	0.028	.318	0.057	.322	0.194	1.771	0.617	.241	0.000	2.012	0.016
Norm. cont. (p.w.)	20	.672	.193		.278		.334		.305		1.782		.282		2.064	
Cond. synth. p.w.	20	.673	.220	0.000	.248	0.000	.360	0.000	.295	0.000	1.796	0.841	.328	0.000	2.124	0.000
Cont. synth. p.w.	20	.671	.189		.274		.317		.341		1.792		.241		2.033	
Alk. ext. III.	20	.668	.220	0.000	.246	0.000	.360	0.000	.310	0.194	1.804	0.617	.318	0.000	2.122	0.000
Alk. ext. cont.	20	.677	.179	0.007	.283	0.028	.324	1.000	.327	0.549	1.790	1.000	.248	0.617	2.038	0.689
Alk. ext. II.	20	.671	.199		.262		.324		.334		1.790		.240		2.030	

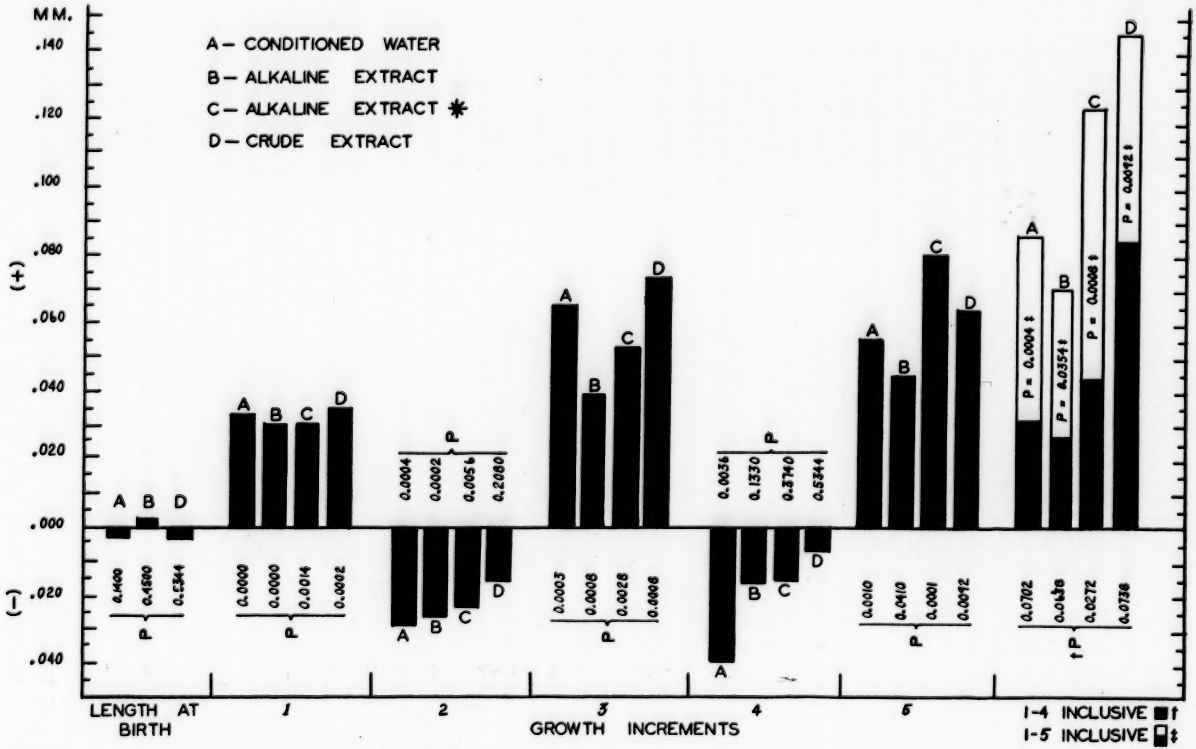
* Norm. = normal; cont. = control; cond. = conditioned; alk. = alkaline; ext. = extract; filt. t.w. = f.t.w. = filtered tap water; synth. p.w. = synthetic pond water; p.w. = pond water.

The second growth increment presents a rather surprising picture. Most of the differences are insignificant, but the general trend is the opposite of that shown during the previous instar. During this instar, the controls showed a greater amount of growth as measured by increase in body length than did experimental groups. This point will be discussed in more detail later. During the third growth period there was a repetition of the trend shown during the first, namely, the experimental animals increased in body length significantly more than their respective controls. The fourth increment resembles the second, i.e., controls increased more in body length than did the experimental groups, although the differences are not statistically significant. A point of interest to the authors was to ascertain whether experimental groups, which had shown two increments (second and fourth) during which their increase in body length was less than their controls, were larger animals or whether they showed, in spite of their excessively rapid growth during two instars, no statistically significant difference from their respective controls in regard to body length. The average size (body length) of these animals is represented in the table as total body length, immediately following the fourth increment. As the data show, the experimental animals of Series III were, with the exception of those reared in conditioned lake waters, significantly greater in body length than their controls. However, when the various series are considered, the picture is not entirely clear since in Series IV, V, and VI, as shown in the table, the differences in total body length after four increments are not generally significant ones, although for the most part the experimental animals are the larger. Further experimentation will be necessary before this point is entirely clear.

Since, physiologically, the first five instars comprise a definite period in that the first brood of young is released at the end of the fifth instar, there was a particular reason to carry the animals through to the end of this interval. It is interesting to note, then, that the fifth growth increment (beginning of the sixth instar) was, in all the experimental groups, in line with the results of the first and third increments.

Series IV, V, and VI confirm to a great extent the results obtained for Series III. These added data clarify one point of importance, namely, for the second growth increment the control groups showed a growth increase which was in each case significantly greater than the increments for their respective test groups. This was not usually true for Series III. The same general alternation of a relatively large increment with a relatively smaller one continues to exist in the case of the test animals of the three latter series. However, even with these added data it is clear that many of the points need further confirmation and clarification.

Even though the results for the separate series bring out certain points of importance, the authors wished to have a composite picture of the effects produced by the several types of treatment. For this purpose the average values, already discussed, for the differences in growth increments between experimental groups and their comparable controls were assembled and averaged. This series of values for the mean of such sets of differences is represented in Figure 1 by the array of histograms for each group and instar. Each column by its height, then, indicates the degree of positive or negative difference when the base line is, in each case, the control appropriate to the experimental group. The degree of statistical significance or nonsignificance is indicated for each column in the graph. In the figure *B*—Alkaline extract includes all groups which were subjected to that type of treatment. In several cases, however, alkaline extracts were used which were old, and the primary purpose of their inclusion was to determine their possi-



AVERAGE GROWTH INCREMENT DIFFERENCES BETWEEN EXPERIMENTAL GROUP AND CONTROL GROUP AVERAGES

* RESULTS OF OLDER PREPARATIONS NOT INCLUDED IN AVERAGES

FIG. 1

ble value after aging. Accordingly, for *C*—Alkaline extract, the data based on the older preparations have been omitted because it was obvious that these extracts had lost, at least partially, their growth-promoting properties. The omitted groups include alkaline extract 1 and 2 of Series V and alkaline extract 2 of Series VI.

As shown in the figure, there is little difference between the averaged body lengths at birth. The increase in length of the first increment was significantly greater for the experimental groups. The results of the second increment are in agreement with those of each individual series, namely, the controls showed greater body-length increases than did the experimental groups. This difference was statistically significant except in the case of the crude extract (*D*).

The regularity of alternation between a relatively large increment one instar and a relatively smaller one the next for the experimental animals is clearly evident in the figure, although in the fourth instar a significant difference occurred only with conditioned water. The last group of histograms (black portion) shows that after four increments the experimental animals were actually larger, although the difference was significant in only one case, that of Alkaline extract—*C*. The others, however, approach significance, and results which would include a greater number of series might possibly demonstrate significance in each case.

The completely black-plus-white histograms in the last group show that when the first five summated increments of each experimental group were plotted against their respective controls, significance was shown in each case. However, this is hardly a valid comparison because it involves three increments (1, 3, and 5) during which increase in size was greater for the experimental groups and only two (2 and 4) increments in which the controls exhibited the greater increase. The results do however indicate that these materials increased total growth during the physiological interval between birth and the release of the first brood of young.

Any attempt to explain the alternation of growth increments evidenced in the results can be at best only tentative. One possible explanation has additional support from the considerable number of observations which the senior author has made on the life-history of *Daphnia*. It has been observed that an extra large number of offspring in a single brood is usually followed by a brood containing a smaller number. Also, critical observations have given the information that an instar of relatively short duration followed by a large growth increment usually results in a smaller increment for the next instar. Possibly these responses have been magnified to some extent by the present experimental treatments and indicate that a refractory period in the following instar is a necessary sequence to a large growth increment. In other words, during an instar which results in a large growth increment there might possibly be an exhaustion of, or interference with, the ability to exercise the growth potential due to accumulated products of metabolism, thus necessitating a period of recovery before the growth potential can again be exercised to its full degree. However, as stated above, this explanation is, although plausible, a hypothesis.

An analogous phenomenon of alternation of growth has been noted by Gause (1934, p. 36). He points out that ". . . . a *Paramecium* which divides rapidly tends to give rise to daughter-cells which divide more slowly, and vice versa." Gause presents, however, no explanation of this effect.

Since *D. longispina* use bacteria as their chief source of food in the experiments it is possible that the conditioned waters and various extracts were effecting a more rapid rate of increase among the bacteria, thus making available a more abundant food supply

for the *Daphnia* and thereby making it possible for them to grow more rapidly. If such was the case, the foregoing experiments did not measure the effect of the various test materials on rate of growth of *Daphnia* but rather the effect of food supply on growth. However, the volume and concentration of the manure-infusion media used for both experimental and control groups was sufficient to supply, presumably, an overabundance of food to the animals at all times. The amount of available food is a very important factor in determining either directly or indirectly the brood size as well as rates of growth (Ingle, Wood, and Banta, 1937), and in the present experiments the numbers of young in the first broods (an average of about 13 per mother) released by the experimental and control animals were very similar to, as well as comparable with, the number ordinarily released by this species under normal laboratory conditions and at a temperature of 25° C. Thus it appears that the test substances were acting directly upon the *Daphnia*.

It might also be argued that the fish-conditioned waters and fish extracts, even though greatly diluted, might serve as an added source of food for the *Daphnia*. In this case, also, the overabundant food supply already present would tend to minimize the operation of this possible factor. In addition to this, it is doubtful whether *Daphnia* are capable of utilizing dissolved foods for their sustenance (Gellis and Clarke, 1935), although colloids are apparently necessary for their development; however, the presence of particulate food of ordinary culture media insures a more rapid development.

SUMMARY AND CONCLUSIONS

1. *D. longispina*, with few exceptions as noted in Table 1, showed a significantly greater growth increment for the first, third, and fifth instars when fish-conditioned water or extracts from the skin of fishes (*C. auratus*) were added to the manure-infusion medium.

2. The second and fourth growth increments of the test groups were smaller than their respective controls; this alternation suggests a refractory period as a necessary sequence to a large growth increment.

3. *Daphnia* of the test groups were, at the time of completion of four growth increments, larger animals (measured by body length), although in most cases not significantly so, but were at the end of five growth increments significantly larger animals in each case than their respective controls.

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AN ANALYSIS OF THE EFFECT OF NUMBERS UPON THE OXYGEN CONSUMPTION OF CARASSIUS AURATUS[1]

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THE investigations reported here represent a continuation of research on an aspect of the mass physiology of animals—the effect of grouping on the activity of fishes (Shlaifer, 1938). Schuett (1934) found that an isolated goldfish, *Carassius auratus* Linné, was more active in an aquarium than were fishes in a group of four in the same volume of water. Escobar, Minahan, and Shaw (1936) confirmed these findings. Breder and Nigrelli (1938) also demonstrated the increased locomotor activity of *C. auratus* when isolated than when in a group of four. There remained, however, the conflicting reports of Schuett (1933) and Bowen (1932) as contrasted with the later work of Schuett (1934) concerning the relation between isolation and oxygen consumption in the goldfish. Schuett's later and more careful work indicated that there was no difference in the amount of oxygen used by a goldfish whether it was isolated or a member of a small group. Shlaifer (1938), measuring the activity of goldfishes indirectly through oxygen consumption and directly through observation, was able to resolve the conflict between studies on locomotor activity and studies on the use of oxygen. He found, among other things, that an isolated animal consumed more oxygen and had a higher rate of locomotor activity than did each fish in a group of two or of four.

The data in the present paper were collected in an attempt to analyze the factor or factors involved in the now well-established "group effect" in *C. auratus* and perhaps thereby to facilitate the analysis of the integrating mechanisms and behavior of fish aggregations in general.

MATERIALS AND METHODS

An extensive description of the general methods used may be found in a previous paper (Shlaifer, 1938). All experiments were performed in a room in Whitman Laboratory which has north windows only; the experiments were conducted between the hours of 9:00 A.M. and 12 noon. At the conclusion of a day's experiments, the fishes were placed in tap water, filtered free of chlorine and sediment, for a 21-hour period of acclimatization. The water was not changed until the conclusion of the experiments on the following day; this eliminated any disturbing effect of handling. Air, bubbled through the aquariums during acclimatization, maintained a normal oxygen tension. With one exception all the animals used were specimens of the common goldfish 5 cm. in length. The animals used for the experiments summarized in A of Table 1 were larger, measuring 8–10 cm. in length; these experiments were performed six months before those summarized in B of Table 1. The technique used throughout was the Rideal-Stewart modification of the Winkler method. The amount of oxygen used is a less direct indicator of activity than is the observation of locomotion. However, its measurement was deemed preferable for several reasons. In the first place, the observational method is not well adapted for sev-

[1] The writer is deeply grateful to Dr. W. C. Allee for his inspiring advice and helpful criticism.

eral of the types of experiments performed—e.g., darkness tests. In the second place, there is good evidence (Shlaifer, 1938) that the measurement of oxygen consumption is free from many of the sources of error that are inherent in the observational technique and is, in general, more sensitive. Possibly it reflects in time such subtle reactions as the movement of the fins or changes in the rate of heart beat caused by stimuli received from other members of the group. Since the activity of individual fishes may vary to some extent and since the activity of any one animal may also vary relatively from day to day, it was necessary to perform a fairly large number of experiments with a large number of animals in order to minimize this source of variation. This was especially true for the oxygen-consumption tests which preclude the measurement of the activity of any single fish in a group; it is also true for experiments utilizing the observational method, since this is less sensitive. In the past investigators along these lines have sometimes made generalizations hardly warranted by the data, considering the known sources of variation. When the oxygen consumption of isolated animals was compared with that of animals in a group of four, equal sampling was effected by performing four times as many experiments on the isolated fishes. These same isolated animals were later used in the groups of four.

EFFECT OF DARKNESS

Among the vertebrates, vision is a particularly important factor in the integration of aggregations, schools, and flocks of fishes and birds. Welty (1934) found that goldfishes become conditioned to run a simple maze more rapidly when grouped than when isolated. The group seemed to stimulate learning behavior through various ways which were all visually integrated. The importance of vision in the schooling of fishes has been shown by Parr (1927) for *Pneumatophorus grex* (Mitchill), by Breder (1929) for *Jenkinsia stolifera* (Jordan and Gilbert), by Spooner (1931) for *Morone labrax* Linnaeus, by Bowen (1931) who found that the closely schooling catfish, *Ameiurus melas* (Rafinesque), failed to aggregate in the dark, and by Breder and Nigrelli (1935) for *Lepomis auritus* Linnaeus.

If vision is a dominant factor in the effect of grouping on the respiratory activity of goldfishes, this effect should disappear in darkness. The experiments described below explore this possibility.

Methods.—Control tests were performed with fishes placed in isolation and in groups of four in ordinary daylight, free from sunlight. At the conclusion of a day's experiments the water was changed, and the aquariums were placed in a dark box which was known to be light tight. The oxygen consumption of the fishes in this experimental condition was measured in a three-hour test period after 21 hours of acclimatization. The same animals were acclimatized and tested for the same period of time in daylight as in darkness. Those tested in isolation were subsequently placed in groups of four, while those in groups of four were later isolated. In the experiments otherwise performed in darkness, the aquariums were removed from the dark box just prior to the test period in order to pour on the seal of mineral oil and to obtain several samples of water for oxygen analysis. This sudden change in illumination may have been a source of artificial stimulation, but was, of course, the same for isolated and grouped animals. An attempt was made to minimize this condition by drawing the shades of all windows and placing a dark cloth over each aquarium until the samples were obtained. Animals that were not in use were kept in a large aquarium in ordinary daylight or under general illumination from ceiling lights when these were on at night.

Results.—Reference to Table 1 shows that in both sets of experiments, A and B, in daylight, the oxygen consumption of the isolated animals is significantly higher than that of fishes in a group of four. This amply confirms previous experiments covering this point (Shlaifer, 1938). In total darkness, on the other hand, there is no significant difference. Thus, it is seen that the "group effect" disappears in the absence of light. Examining the data further, we find that in both A and B the oxygen consumption of fishes in a group of four is not significantly higher in daylight than in darkness. Isolated animals, however, have a distinctly higher rate of oxygen consumption in the light. Thus, the

TABLE 1

EFFECT OF DARKNESS ON THE AMOUNT OF OXYGEN CONSUMED PER FISH BY GROUPED AND ISOLATED GOLDFISHES

Experimental Condition	Test Period (Hours)	I. One Fish			IV. Four Fishes		
		No. of Cases	Mean Cc. O_2 Consumed per Fish		No. of Cases	Mean Cc. O_2 Consumed per Fish	
			Daylight	Darkness		Daylight	Darkness
A*, §, ‡	3	32×1	7.44	6.10	8×4	6.01	5.70
B§, ‡, †	3	32×1	3.82	2.90	8×4	2.72	2.60

* = 8–10-cm. fish. † = In 7.5-liter aquarium. ‡ = In 6 liters of water. § = 5-cm. fish.

TABLE 1*a*

STATISTICAL ANALYSIS OF TABLE 1 SHOWN IN *P*-VALUES*

Experimental Condition	I vs. IV—Light	I vs. IV—Dark	I—Light vs. I—Dark	IV—Light vs. IV—Dark
A	0.026	0.590	0.0002	0.380
B	0.0048	0.406	0.0000	0.264

* Upper limit of statistical significance is set at 0.05. This is three times the probable error. P = 0.01 indicates good significance, while a value of 0.100 or more indicates little significance ("Student," 1925).

group effect appears to be eliminated not through increased use of oxygen by grouped fishes in the dark but by the decreased consumption of the isolated animals in that state.

These data indicate the importance of vision as a factor in the lessened activity of grouped goldfishes.

EFFECT OF BLINDNESS

If the group effect is lost in total darkness, indicating a role of vision, it follows that the same results should obtain with blinded fishes. Parr (1927) found that blinded individuals did not mill or exhibit schooling reactions. Bowen (1931) found that blinded catfishes did not aggregate.

Methods.—A group of individuals from set B of the darkness tests were blinded by completely removing both eyes. Though more drastic, this method seemed to be prefer-

able to covering the eyes with opaque materials which might wear off. Several animals did not survive the operation, and only those were used which, except for their blindness, exhibited normal behavior, at least as judged by the observer. The fishes that died did so in a few days; the survivors were observed for several weeks before they were used. These animals lived for months after the experiments were completed, indicating excellent recovery from the operation. The blinded fishes were placed alternately in isolation and in groups of four in daylight, and their oxygen consumption was measured in a test period each day. Removal of their eyes does not permit the use of the experimental animals as control animals every other day as was possible in the tests in darkness. However, it should be borne in mind that before being blinded, these animals had been used in the preceding series of experiments and, when in ordinary daylight, showed a definite group effect.

Results.—The data in Table 2 indicate that when fishes are blinded, there is no significant difference in the oxygen consumption of grouped and isolated animals. When compared with the fishes placed in darkness in B of Table 1, the blinded animals, themselves selected from set B, are seen to have a lower rate of oxygen consumption in both the isolated and the grouped states. This is partially a result of the fact that the surviving blinded animals were somewhat smaller than those in the original group. Thus, the elimination of the action of vision through blinding also eliminates the group effect.

TABLE 2

EFFECT OF BLINDNESS ON THE AMOUNT OF OXYGEN CONSUMED BY GROUPED AND ISOLATED GOLDFISHES*

Isolated		Group of Four		Mean Difference	P
No. of Cases	Mean Cc. of O_2 Consumed per Fish	No. of Cases	Mean Cc. of O_2 Consumed per Fish		
28×1	2.20	7×4	2.07	0.13	0.7186

* Test period, 3 hours; volume, 6 liters of water in a 7.5-liter aquarium.

VISUAL CONTACT

Though the darkness and blindness experiments show that the sense of vision is important, they do not rule out the possibility of other sense organs coming into play in the group behavior of the goldfish in an important though perhaps subtle manner. It is possible that vibrations of tails of fishes set up pressure disturbances, detected perhaps by the lateral-line sense organs, which influence the activity of grouped animals. Thus, in the strongly thigmotactic *A. melas* Bowen (1931) demonstrated such responses and touch as well in the aggregating behavior of this species. Even with these bullheads vision was found to be essential for aggregations since blinded fishes and normal ones kept in the dark did not aggregate.

The experiments reported below were designed to eliminate all factors but vision. If isolated goldfishes manifest a group effect when in contact through vision alone with

other goldfishes, it may be concluded that vision is a very important factor in their normal group behavior.

Methods.—A 3.5-liter aquarium with dimensions of 17.5 cm. by 13 cm. and 20 cm. deep was placed in a 15-liter aquarium whose dimensions were 30 cm. by 27 cm. and 24 cm. deep. The walls of both aquariums were of transparent glass. The smaller aquarium was filled with 2.5 liters of water which gave a depth of 13 cm. at the mark. The larger aquarium was filled with enough water, about 6 liters, so that, when the smaller one was placed in it, the two water levels coincided. On each of the four vertical sides there were 5 cm. of space between the inner surface of the large aquarium and the outer surface of the small aquarium. One goldfish was placed in the smaller aquarium. In the control tests there were no animals in the outer aquarium, while in the experimental condition there were three goldfishes present. The animals used in all experiments were rather small—5 cm. in length; this permitted sufficient free movement in either aquarium. The usual oxygen seal of mineral oil was placed on the water surface of

TABLE 3

EFFECT OF VISUAL CONTACT WITH OTHERS OF THE SAME SPECIES ON THE AMOUNT OF OXYGEN CONSUMED BY ISOLATED GOLDFISHES*

CONTROL†		EXPERIMENTAL‡		MEAN DIFFERENCE	*P*
No. of Cases	Mean Cc. of O_2 Consumed	No. of Cases	Mean Cc. of O_2 Consumed		
16	1.99	16	1.32	0.67	0.0002

* Test period, 3 hours; volume, 2.5 liters of water in a 3.5-liter aquarium.
† Isolated fish in no visual contact with other fishes.
‡ Isolated fish in contact through vision alone with three other fishes.

the small aquarium only. The oxygen consumption of the isolated animal in the small aquarium was measured each day. The isolated fish was alternated daily between complete isolation and contact through vision alone with three other animals of the same species. Several fishes were tested in the course of the experiments. One might be inclined to doubt whether the optical powers of the goldfish are sufficiently acute to enable the isolated animal in the foregoing setup to respond to its fellows in the outer aquarium. However, Welty (1934) in the course of his experiments on learning in the goldfish showed that these animals learn to swim a simple maze more rapidly after having seen, through transparent glass, other fishes perform in it.

Results.—Table 3 shows a significant decrease in the oxygen consumption of the isolated fish in the inner aquarium when in visual contact with three others of its kind.

Since all other stimuli but those due to vision have been eliminated, this experiment indicates that visual response to other fishes is a dominant factor responsible for the group effect in goldfishes which is indicated by the decreased rate of activity and oxygen consumption in a group of four as contrasted with similar data for isolated animals.

EFFECT OF MIRROR IMAGES

This experiment was designed to investigate further the role of visual stimuli in aggregations of *C. auratus*. Also, it furnishes an interesting sidelight on general fish be-

havior. Spooner (1931) found that the bass—*Morone labrax*—a vigorous fish with a rather strong tendency to school, gave definite reactions to a mirror. An isolated fish in contact with a mirror would often lie motionless against it for extended periods of time. When Spooner (1931) used mirror surfaces that were broken up by running elastic bands across the surface of the mirror at definite intervals, the fishes reacted distinctly to these mirrors if the bands were three-quarters of an inch apart; at smaller intervals there were either weak reactions or none at all.

Methods.—7.5-liter aquariums containing 6 liters of water were used. The aquariums used for the control tests had four transparent vertical glass sides; those used for the experimental condition were mirrored on two contiguous vertical sides which formed a right angle. The remaining two vertical sides of the mirrored aquariums, also forming a right angle, were constructed of transparent glass. Isolated animals were alternated each day between the plain and mirrored aquariums, and their oxygen consumption was measured in a 3-hour test period.

TABLE 4

EFFECT OF MIRROR IMAGES ON THE AMOUNT OF OXYGEN CONSUMED BY ISOLATED GOLDFISHES*

CONTROL†		EXPERIMENTAL‡		MEAN DIFFERENCE	P
No. of Cases	Mean Cc. of O_2 Consumed	No. of Cases	Mean Cc. of O_2 Consumed		
22	2.92	22	2.09	0.83	0.0000

* Test period, 3 hours; volume, 6 liters of water in a 7.5-liter aquarium.
† Isolated fish in a clear-sided aquarium.
‡ Isolated fish in an aquarium mirrored on two adjacent sides.

Results.—The data in Table 4 show a significant fall in the oxygen consumption of the isolated fish when in the mirrored aquarium. All factors but vision have again been eliminated. The animal in the mirrored aquarium tended to remain near and parallel to the mirrored surfaces, apparently reacting as though it was lining up with another fish in a group of two.

The experiments described above leave little doubt as to the important role played by sight in these group reactions and make unnecessary the analysis of other factors at this time. Granted that this sense is so important in the responses tested, how does it act? The fishes may be reacting to (*a*) color, (*b*) form, (*c*) type of movement, (*d*) amount of movement, or (*e*) some combination of these factors.

REACTION TO COLOR

The response of fishes to colors is a field which still requires investigation. In a comprehensive review Warner (1931) criticized the lack of control of the intensity factor and was of the opinion that most of the experimental work would bear repeating. White (1919, 1927) demonstrated that mud minnows and sticklebacks can discriminate wavelengths and not merely intensities of light. Noble and Curtis (1935) found that when the female cichlid, *Hemichromis bimaculatus*, was given a choice between a male with ery-

throphores expanded by yohambine and a paler control, she usually selected the former. Brown (1937) demonstrated the reaction of the largemouthed black bass, *Aplites salmoides*, to colors.

Methods.—The aquarium setup was the same as that described for the visual-contact experiments. In the control setup three normal orange-colored goldfishes were placed in the large aquarium. In the experimental condition, these animals were replaced by three goldfishes of the same size and shape and of a dark gray or black color, mixed with a slight trace of orange. In general, at least to the human eye, the experimental animals formed a distinct contrast to those used in the control tests. Isolated normal orange-colored goldfishes in the smaller aquariums were alternately placed in visual contact with the orange and with the dark animals, while their oxygen consumption was measured as usual.

Obviously, these experiments were not designed to test the reactions of *Carassius* to color as such. Rather, they are naturalistic in nature and do explore the possibility that orange goldfishes respond differentially to differently colored members of their species.

TABLE 5

EFFECT OF VISUAL CONTACT WITH ORANGE OR WITH DARK INDIVIDUALS ON THE OXYGEN CONSUMPTION OF ISOLATED GOLDFISHES*

Control†		Experimental‡		Mean Difference	*P*
No. of Cases	Mean Cc. of O_2 Consumed	No. of Cases	Mean Cc. of O_2 Consumed		
24	1.71	24	1.61	0.10	0.363

* Test period, 3 hours; volume, 2.5 liters of water in a 3.5-liter aquarium.
† Isolated fish in visual contact with three normal orange goldfishes.
‡ Isolated fish in visual contact with three darkly colored goldfishes.

Results.—Reference to Table 5 shows that there is no significant difference in the rate of oxygen consumption of an isolated fish when it is in visual contact with a goldfish of a distinctly darker color than the normal orange type. Thus, the major visual response of fish to fish is apparently not in terms of color. It may be that the technique and experimental setup were not sensitive enough, but the general indications are that other factors are involved.

REACTION TO BODY FORM AND MOVEMENT

The factors that remain to be analyzed are the reactions to body form and to movement. Spooner (1931) found that a fish will be attracted to and will lie alongside a dead fish supported by glass in the position of a resting live one. Thus it is seen that a fish may be attracted by the form of another one that is completely devoid of movement. Apparently, the form in that case had to be a good replica of the living animal, for, when rough models of fishes were tried, no attraction was obtained.

The following experiment was designed to test the factors of form and movement simultaneously.

Methods.—The aquarium setup was again the same as that described for the visual-contact tests. Normal orange goldfishes were killed and injected with formaldehyde and

were mounted on sharply pointed glass rods attached at the base to hidden wire loops for support. These mounted animals were placed in the outer aquarium in the normal swimming position close to and parallel to the sides of the smaller aquarium. The fishes in both aquariums in that type of setup usually swim in a horizontal plane about 4 cm. above the bottom. Accordingly, the mounted animals were also placed at that level. No odor of formaldehyde, which had been injected into the dead animals, could be detected when they were in position in the aquarium. Three mounted fishes were placed in the larger aquarium in the experimental setup, two of them, nose to tail, next to one long side of the small aquarium; the third was placed in the opposite passage way and pointed in the same direction as the other two. When it was thought that the color of the mounted specimens was not quite normal, they were replaced by newly killed and mounted animals. The hollow transparent glass rods on which the fishes were mounted probably eliminated any possibility that the animal in the inner aquarium would respond to the mounting material as well as to the mounted fish. The isolated test animals in the inner

TABLE 6

EFFECT OF VISUAL CONTACT WITH NORMAL OR WITH MOUNTED INDIVIDUALS ON THE OXYGEN CONSUMPTION OF ISOLATED GOLDFISHES*

CONTROL†		EXPERIMENTAL‡		MEAN DIFFERENCE	P
No. of Cases	Mean Cc. of O_2 Consumed	No. of Cases	Mean Cc. of O_2 Consumed		
20	1.95	20	1.46	0.49	0.0001

* Test period, 3 hours; volume, 2.5 liters of water in a 3.5-liter aquarium.
† Isolated fish in visual contact with three normal goldfishes.
‡ Isolated fish in visual contact with three dead and mounted goldfishes.

aquariums were alternately placed in visual contact with the control setup of three normal orange goldfishes and with the experimental condition of the three killed and mounted specimens; their oxygen consumption was measured as usual in a 3-hour test period.

Results.—The data in Table 6 show that the oxygen consumption of the isolated fish is significantly lower when that animal is in visual contact with immobilized fishes. The interesting implications of this experiment are too lengthy to be treated here and are reserved for the following section.

DISCUSSION

The effect of grouping on the oxygen consumption and iocomotor activity of fishes is an aspect of animal aggregations. Accordingly, as a somewhat minor result of these experiments, it is possible to suggest the place occupied by *C. auratus* in a system of classification of such aggregations. This becomes more interesting in connection with the fact that ". . . . no hard and fast line can be drawn between well-integrated social organizations and loosely integrated aggregations which are usually regarded as being definitely non-social" (Allee, 1931, p. 14), since the goldfish is not a closely schooling species. Presumably, therefore, it occupies a low position even in the more inclusive estimates of the extent of sociality such as those given by Wheeler (1928) and Allee (1938).

In the system of classification of animal aggregations given by Allee (1931, p. 35, 36), the aggregating behavior of the goldfish might conceivably fall into the two major divisions given for individuals which are not organically connected: (1) aggregations primarily due to reactions to the environment and (2) aggregations primarily due to reactions to other organisms.

Theoretically, goldfish aggregations that fall into the first category may occur in nature. Thus, the animals may be attracted tropistically to some particular region of a pond that at a particular time presented optimal chemical or physical conditions. This type of aggregation is much less likely even in large aquariums or tanks in the laboratory; in smaller aquariums under controlled experimental conditions, it may practically be eliminated from consideration. For example, it is observed that goldfishes in a group of four may tend to occupy, more or less, a certain region in an aquarium, the region occupied being changed from time to time. When the animals are blinded, they are distributed at random indicating that the reaction was a result of integration with other individuals rather than caused by some differential factor in the environment.

Aggregations of goldfishes in nature probably fall much of the time in class 2; in laboratory aquariums, properly protected from any changes in the uniformity of the environment such as sunlight, proximity to heat, etc., category 2 would be the sole possibility.

In addition to the foregoing subdivisions of aggregations based on the method of integration, Allee (1931, p. 36) lists the following subdivisions based on the degree of integration:

1. Relatively slightly integrated groups in which the primary (individual) reactions predominate and whose survival value is apparent only after experimentation.

2. Moderately well-integrated groups in which the secondary (group) reactions predominate, although primary reactions are still strongly in evidence, and whose survival value is more obvious.

3. Highly integrated groups in which the primary reactions are decidedly in the minority and the social value is strongly in evidence.

Aggregations of *C. auratus* fall nearer to class 1 than to class 2. The closely schooling mackerel would fall definitely into class 2.

Shlaifer (1938) found that a goldfish has a higher rate of oxygen consumption and locomotor activity when isolated than when it is a member of a freely moving group. The experimental data presented here confirm these earlier results and extend them to demonstrate the importance of visual stimuli in decreasing the activity of grouped goldfishes. Visual contact of an isolated animal with freely swimming goldfishes or even with a mirror self-image retards activity. This is found to be independent of the color. The data listed in Table 6 also show that the activity of the isolated animal is greater if the fishes in the visual field are moving than if they are immobile. Therefore, the isolated goldfish in visual contact with the group of immobilized animals is affected in its behavior even more than if the fishes in its visual field are active. Under these conditions the isolated animal can be reacting to form and to color only. Since color, within the limits tested, is not needed to produce the usual group effect, it appears that, under the conditions tested, the isolated fish is reacting to form alone. Apparently, it is enough that a goldfish see its own mirror image or an immobilized goldfish in order to undergo the quieting effect of the group. The extent to which this form can be altered from that of goldfishes and still evoke a similar effect remains to be discovered by direct experimentation.

It was shown in the darkness experiments that the group effect is eliminated not

through an increase in oxygen consumption by the grouped fishes in the dark but by decreased consumption by isolated animals in that state. If we assume that darkness tends to act as a quieting factor for goldfishes, an explanation may be offered for the observed results as follows: The isolated animal, subjected to no stimuli from other fishes, is affected only by the change in illumination, and its activity is diminished; in the grouped animals, the quieting effect of darkness is equalized by the loss of visual inhibition of activity and, perhaps, by random contacts.

The dominance of vision as the factor that inhibits activity in groups of goldfishes can be further confirmed by comparing certain data in Tables 1 and 3. These experiments were selected for comparison because they are the only ones whose general conditions are similar enough for that purpose. Even these experiments were performed under setups that were sufficiently different to make impossible a perfect comparison. Thus, the oxygen consumption of the fishes in Table 3 is lower, regardless of the condition of grouping, than is that of the animals in B of Table 1; this is probably due to the distinctly lower volume of water available to the animals in the smaller aquarium used for the experiments summarized in Table 3. (A of Table 1 is not used in the comparison because the individuals in those experiments were considerably larger than those used later.) It is seen in B of Table 1 that the oxygen consumption of an isolated fish in daylight is decreased by 29 per cent when it is subsequently used as a member of a group of four. Reference to Table 3 shows that, when an isolated fish is made a member of a group of four through visual contact alone, its oxygen consumption is reduced by a similar amount—33 per cent. Thus, visual response to other fishes, taken alone, may produce a group effect of the same order of magnitude as does that shown when an individual is an actual member of a group of four.

Of the four factors involved in visual response—namely, color, form, type of movement, and amount of movement—only form has been found to act as an inhibitor of activity. The data in Table 6 indicate that inhibition of activity of an isolated goldfish is greater when it responds through sight to form *minus* movement than it is when the animal responds to form *plus* movement. This may mean that movement as such is a stimulator of activity. The role of the type of movement in aggregations of fishes is a much more subtle affair and may be investigated through the use of heterotypic groups.

Experiments on fishes in laboratory aquariums suffer from the criticism of being divorced from conditions as they occur in nature. However, the obvious necessity for the careful control of variables greatly mitigates this criticism. Apropos of this consideration, the experiments described in this paper may be listed in the following increasing degree of artificiality: (*a*) experiments with normal fishes in darkness, (*b*) experiments with normal fishes in daylight, (*c*) experiments with blinded fishes, (*d*) effect of mirror images, and (*e*) contact through vision alone with (1) normal fishes and (2) mounted fishes.

Many reactions of animals tested experimentally may never occur, as far as we know, in a natural environment. Nevertheless, it is important to know not only the normal behavior that usually is manifest but also the behavior potentialities of an organism. Very often it is the analysis of behavior under unnatural conditions which are carefully controlled that facilitates the explanation of normal behavior. Also, many factors in the behavior complex of an organism are so subtle as to necessitate artificial methods of analysis if they are to be discovered at all.

As discussed above, it is apparently the visual perception of body form that inhibits

the activity of grouped goldfishes. Granted that this is so, it is difficult to offer an explanation for this pattern of behavior. Breder and Nigrelli (1935) state that a schooling fish primarily holds a position of maximum "comfort" by fixing its vision on some object, either a stationary one or a moving one; this difference determines whether the fish maintains a stationary position or swims forward. If this also holds true for the goldfish, which is not a strongly schooling animal, it may be that the increased activity of the isolated animal, which cannot find an object on which to fix its vision, is a result of a state of "psychic unrest," a state which may well defy direct experimental exploration. Thus, as in most aspects of animal behavior, the ultimate analysis of the *modus operandi* remains as elusive as it is interesting.

One may speculate as to whether the reaction of a goldfish to the body form of others in a group is a response to the particular type of body form characteristic of the species or to body form of fishes in general. Escobar, Minahan, and Shaw (1936) worked with a heterotypic group of four fishes; the species used were *C. auratus*, *Gambusia affinis*, *Oryzias latipes*, and *Macropodus verdi-auratus*. The group effect found here for the goldfish was the opposite of that found for it in homotypic groups of four. The activity of the goldfish in the heterotypic group exceeded even that in an isolated condition. More extensive work with heterotypic groups in observing locomotor activity and measuring oxygen consumption and utilizing, perhaps, some of the experimental setups described above may yield fruitful and interesting results which would have significance for the general problem of the integration of animal aggregations. It is quite probable that any group effect that might be found in such heterotypic groups would be produced by a combination of factors distinctly more complex than is to be found in the homotypic groups used so far. Thus, the color, form, type of movement, and amount of movement might vary considerably when several species are used. The activity of a goldfish in heterotypic groups of three species might in some cases be found to be a resultant of two opposing tendencies caused by the other two species in the group. Perception of body form might stimulate activity in heterotypic groups, or its inhibitory action might be masked by some more dominant responses to type and amount of movement. It would be necessary, of course, to employ suitable techniques to isolate, as far as possible, the variables in this complex. Any conclusions drawn from the behavior of fishes in heterotypic groups would have to be based on a considerably larger group of data than is necessary for homotypic groups because of the variables involved. However, the implications, especially at the relatively low state of integration that is involved in such aggregations, appear to justify the work that would be necessary. Such an analysis is the more important because heterotypic aggregations have ecological implications of far-reaching significance in the study of animal communities in nature. Experiments along these lines are now under way in our laboratory.

Parr (1937) makes the interesting point that a fish with a rather rigid body—the mackerel, for instance—displays definite schooling behavior visually integrated, though it is incapable of self-perception. Therefore, Parr (1937) states: ". . . . the reaction of the mackerel to the perception of others of its kind cannot be in terms of a response to individuals recognized to be similar to itself, but must be based upon an automatic association mechanism of a different order in which the perception of another fish similar to itself fits as a key fits into a lock." If this is true for the mackerel, it also holds for *C. auratus*. From this point of view, the proposed analysis of heterotypic aggregations of fishes presents new and interesting problems.

SUMMARY

1. The effect of numbers upon the oxygen consumption of grouped and isolated goldfishes, manifested by a decreased rate of oxygen use in the group, is lost when the animals are placed in the dark or are blinded.

2. The oxygen consumption of isolated goldfishes is decreased when they are placed in contact through vision alone with others of the same species, with their own mirror images, or with recently killed and mounted forms.

3. It is evident that vision is the major sense involved in this aspect of mass physiology.

4. Inhibition of activity in grouped animals is probably due to the perception of the form rather than the color or movement of the fishes comprising the group.

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A PERFUSING SOLUTION FOR THE CRAYFISH HEART AND THE EFFECTS OF ITS CONSTITUENT IONS ON THE HEART[1]

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A SATISFACTORY solution for nerves of the crayfish, *Cambarus clarkii*, was developed by van Harreveld (1936) based upon chemical analyses of blood. Since its composition varied considerably from solutions reported by others to be satisfactory for crayfish, especially with respect to calcium, it seemed desirable to test it on the crayfish heart, to compare it with those other solutions, and to study the effects of constituent ions on the heart.

TABLE 1

COMPOSITION AND CATION RATIOS OF SOME OF THE SOLUTIONS USED ON THE CRAYFISH HEART

Solution	Total Molarity	NaCl Molar	KCl Molar	$CaCl_2$ Molar	$MgCl_2$ Molar	$NaHCO_3$ Molar	Cation Ratios When Na = 100*		
							K	Ca	Mg
Crayfish blood (van Harreveld)	Δ° C. = 0.85	.....	0.0051	0.0137	0.0028	.....	.....	.....	.....
van Harreveld	0.2288	0.2051	0.0054	0.0135	0.0026	0.0022	2.63	6.58	1.27
Lindeman	0.1768	0.1709	0.00375	0.00216	.....	.....	2.19	1.26	.....
C	0.1992	0.1880	0.00402	0.00720	.....	.....	2.14	3.83	.....
Hoffmann	0.228	0.2200	0.004	0.002	.....	0.002	1.82	0.91	.....

* Omitting the Na in $NaHCO_3$.

The composition of van Harreveld's solution is given in Table 1. The pH varied from 7.2 to 7.8, and the depression of the freezing point was −0.86° C. which agrees well with the depression for crayfish blood (−0.85° C.; van Harreveld, 1936). This solution was first compared with the one reported by Lindeman (1929); then with a solution having intermediate amounts of sodium and calcium chlorides (solution C); with double Ringer's solution, which Hoffmann reported he used, although the compositions given are incorrect (Hoffmann, 1912–13); with isotonic single salt and glucose solutions, and with thirty-one other salt mixtures, the ionic ratios of which covered many significant possibilities.

[1] Nearly all the experiments herein reported were done at the Kerckhoff Laboratories in the fall and winter of 1937. To Drs. T. H. Morgan, C. A. G. Wiersma, and to other members of the staff the senior author expresses sincere thanks for the privileges and courtesies extended to him.

Since the extensive report of Plateau (1880) on the heart of decapods, there have been published many accounts of attempts to perfuse the heart of crustacea and of a few other invertebrates in order to compare the cardiac behavior of vertebrates with that of invertebrates. The work of Carlson (1905, 1906, 1906–7) on *Limulus* is perhaps the best known. A great variety of perfusing solutions has been used, and, as might be predicted, conflicting and equivocal results have been obtained, so that the question of how the invertebrate heart differs from the typical vertebrate heart is still largely unanswered. One probable reason for confusion has been the frequent use of unbalanced and improperly antagonized perfusing solutions. In the experiments reported here it has become clear that for the crayfish heart a favorable perfusing solution must correspond, in its inorganic composition at least, to the crayfish blood. It is likely that the same thing will be found true of other invertebrates. A critical review of the literature will, therefore, be postponed until proper solutions have been developed for representative species.

METHODS

Crayfish of both sexes collected in the vicinity of Pasadena and varying in body length from 6 to 10 cm. were prepared for perfusion of the heart as follows. The larger part of the carapace between the eyes and the cervical grooves was removed. The stomach, "liver," and reproductive glands were then removed, with the animal held vertically, head downwards.

Alimentary or liver juices were found to be highly injurious if they came in contact with the heart. The cavity was thoroughly washed with the physiological solution, and the ganglionic nerves were severed by cutting along each side of the ganglionic chain directly under the pericardial region. The anterior section of the pericardium was then cut away, thus exposing the pericardial cavity. A small spring pinch clamp 8 mm. long, made of rustless steel wire, was fastened to the midanterior margin of the heart. The animal was securely fastened in a vertical position head upwards. A thread attached the pinch clamp to a heart lever using a fine human hair as a marker on a lightly smoked drum. The tip of a glass tube with bore of about 1.5 mm. and a funnel-shaped opening at the upper end, was fastened about halfway down in the pericardial cavity at one side of the heart. The experimental solution flowed into that tube at a constant rate (6 ± 1.0 ml./min.) and at room temperature ($22° \pm 2.0°$ C.), filling the pericardial cavity and overflowing around the top of the latter. This method of perfusion was found to be far preferable to methods in which the crayfish is held in a horizontal position. Different perfusing solutions at the same rate of flow could quickly be substituted for van Harreveld's solution and compared with it. Clock time and frequency of beat, measured by a stop watch, were recorded on the smoked paper along with the heart record.

RESULTS

During the first few minutes of perfusion the frequency, tone, and amplitude of the heart beat varied considerably, but by the end of 15 minutes they usually became relatively constant. Of eighty-two animals used for perfusion by van Harreveld's solution, the normal rate at the end of 15 minutes varied from 62 to 118 beats per minute with an average of about 93. The heart beat of seven intact animals observed through Cellophane windows, between 20° and 23° C., varied from 75 to 136, with an average of 116 per minute. In a large percentage of the preparations, the beat was extremely regular, and the amplitude of successive beats was constant. A few animals showed constant but

different amplitudes of alternate beats or rhythmic beats of more than two amplitudes or a continuous irregularity of rate and amplitude. Such hearts rarely ever became typical afterward.

Hearts perfused only with van Harreveld's solution continued normal beating for many hours (usually from 8 to 10 hours; occasionally from 9 to 14 hours, and a few from 18 to 20 hours). With occasional stimulation by lentin (carbamylcholine chloride) two hearts continued beating for 24 and 29 hours. There is little doubt that the other tissues of the animal were dead within 3 hours. It is believed that injury to the heart by the operation and by the pinch clamp is the most common cause of failure of hearts perfused only by that solution, if failure occurs before 5 hours. Since the solution contains no nutritive materials, no hemocyanin, and none of the other organic constituents of crayfish blood, it is not surprising that the heart stops eventually. Such arrest occurs in diastole and is characterized during the later hours by slowly decreasing frequency and amplitude without any significant change in tone.

Fifteen hearts were perfused only with Lindeman's solution, and in each case the frequency exceeded that found for van Harreveld's by from 10 to 50 per cent. Beating continued for much shorter periods of time (usually less than 2 hours; occasionally from 2 to 3 hours). Arrest was characterized by slight increase in tone, as well as by decreased frequency and amplitude. If hearts were first perfused by van Harreveld's solution and then changed to Lindeman's, marked increase in frequency and amplitude always occurred, followed by decreases in each, up to arrest in tonic diastole or one-half systole. If the solution was replaced by van Harreveld's before the frequency had decreased too much, the heart quickly recovered its normal characteristics. Obviously Lindeman's solution is much less satisfactory for the heart than van Harreveld's. The former contains no magnesium, is not buffered by bicarbonate, has a molarity 23 per cent less than the latter, and contains only one-fifth the latter's ratio of calcium ions to sodium ions. Results to be described later show that the absence of magnesium is relatively unimportant and that the lack of buffer action and the lower osmotic pressure are minor faults. The chief factor, therefore, in rendering Lindeman's solution unsatisfactory is the incorrect calcium content.

Solution C, with intermediate amounts of sodium and calcium and an intermediate molarity, also caused increased frequency and amplitude as compared with van Harreveld's, but to a lesser degree than Lindeman's. Furthermore, there was considerable adaptation, in that the frequency and amplitude returned to normal in some cases, and beating continued for from 2 to 4 hours. Double frog Ringer's solution (or Hoffmann solution, cf. Table 1), with the same molarity as van Harreveld's but with smaller amounts of potassium and calcium than Lindeman's, was found to be very unsatisfactory, causing marked increase in frequency and amplitude at first, followed by rapid decrease up to arrest in systole within 30 minutes. Varying the pH of van Harreveld's solution from 6.4 to 8.4 caused no significant changes in the frequency, tone, or amplitude of the heart beat. Below or above those limits changes in tone and amplitude usually occurred, although a detailed study of such changes was not made.

Contradictory results were often obtained during the early experiments, until it was discovered that the heart's behavior in the solutions might depend upon previous treatment. For example, if a beating heart which had been perfused with double Ringer's solution for a certain period was returned to van Harreveld's solution, temporary arrest in diastole might occur, although recovery to normal would usually follow within the

next hour. Subsequently, therefore, all results were checked on fresh hearts treated previously only with van Harreveld's solution.

To determine the relative roles of each cation and the best mixture of them, various significant combinations, the majority of which are displayed in Table 2, were tested. In all of the solutions the pH was practically the same owing to the buffering action of

TABLE 2

IONIC RATIOS, MOLARITIES, AND CHARACTERISTICS OF SOLUTIONS USED ON THE CRAYFISH HEART

Solution	NaCl	KCl	$CaCl_2$	$MgCl_2$	$NaHCO_3$	Total M	Characteristics
van Harreveld's	0.205	0.0054	0.0135	0.0026	0.0022	0.2288	"normal"
1	0.217	0.0057	.....	0.0026	0.0022	0.2275	No Ca
2	0.210	.....	0.0138	0.0027	0.0022	0.2287	No K
3	0.207	0.0054	0.0136	.....	0.0022	0.2282	No Mg
4	0.208	0.0046	0.0114	0.0026	0.0022	0.2288	−15% K
5	0.204	0.0063	0.0134	0.0026	0.0022	0.2285	+17% K
7	0.201	0.0098	0.0132	0.0025	0.0022	0.2287	+84% K
8	0.195	0.0051	0.0238	0.0025	0.0022	0.2286	+82% Ca
9	0.191	0.0093	0.0234	0.0024	0.0022	0.2283	+84% K, +74% Ca
10	0.188	0.0122	0.0232	0.0024	0.0022	0.2280	+125% K, +74% Ca
11	0.206	0.0054	0.0125	0.0026	0.0022	0.2287	−7% Ca
12	0.203	0.0054	0.0147	0.0025	0.0022	0.2278	+9% Ca
13	0.188	0.0100	0.0260	0.0024	0.0022	0.2286	+85% K, +92% Ca
14	0.214	0.0027	0.0068	0.0027	0.0022	0.2284	−50% K, −50% Ca
17	0.202	0.0053	0.0160	0.0026	0.0022	0.2281	+18% Ca
18	0.205	0.0054	0.0128	0.0026	0.0022	0.2280	−5% Ca
19	0.1025	0.0027	0.0067	0.0013	0.0022	0.1154	½ v.H.
20	0.410	0.0108	0.0270	0.0052	0.0022	0.4552	2×v.H.
21	0.207	0.0027	0.0136	0.0026	0.0022	0.2281	−50% K
22	0.209	0.0041	0.0103	0.0026	0.0022	0.2282	−24% K, −21% Ca
23	0.208	0.0055	0.0103	0.0026	0.0022	0.2286	−21% Ca
24	0.212	.....	0.0139	.....	0.0022	0.2281	Na+Ca only
25	0.220	0.0057	.....	.....	0.0022	0.2279	Na+K only
26	.....	0.058	0.145	.....	0.0022	0.2052	Ca+K only
27	0.198	0.0053	0.0200	0.0025	0.0022	0.2280	+53% Ca
28	0.196	0.0080	0.0200	0.0024	0.0022	0.2286	+48% K, +48% Ca
29	0.194	0.0104	0.0200	0.0023	0.0022	0.2289	+92% K, +48% Ca
30	0.182	0.0240	0.0180	0.0022	0.0022	0.2284	+344% K, +33% Ca
31	0.187	0.0246	0.0123	0.0024	0.0022	0.2285	+350% K
32	0.191	0.0201	0.0125	0.0024	0.0022	0.2282	+270% K
33	0.198	0.0052	0.0130	0.0100	0.0022	0.2284	+284% Mg
34	0.189	0.0050	0.0125	0.0196	0.0022	0.2283	+650% Mg

the sodium bicarbonate, the concentration of which was constant. Also, with the exception of solutions 19 and 20, osmotic pressure was essentially the same, since the total molarities were relatively constant.

The absence of magnesium from van Harreveld's solution (solution 3) produced no significant effect for short periods up to 2 hours; after that time, decreases in frequency and increases in amplitude occurred at a greater rate than with the whole solution. Obviously, the magnesium ion plays a relatively minor role in sustaining and regulating the heart beat of crayfish. Absence of calcium (solutions 1 and 25), however, caused quick

arrest in systole; absence of potassium (solutions 2 and 24) or of sodium (solution 26) caused quick arrest in diastole. Conversely, isotonic calcium chloride or magnesium chloride caused arrest in diastole, while isotonic sodium chloride or potassium chloride or sodium bicarbonate caused arrest in systole. These results, in some respects, are similar to those reported by Walzl (1937) for the oyster but differ considerably from those of Lindeman (1928) on the crayfish. Since the latter's solution was so incorrectly balanced for calcium, the results obtained by changing from it to other solutions were undoubtedly abnormal results of hearts treated earlier with unbalanced solutions as previously described.

Isotonic sodium chloride regularly caused an immediately increased amplitude and frequency for from 1 to 5 minutes, accompanied by a gradually increased tone and decreased amplitude. During the next few minutes the frequency slowly decreased while the tone increased and the amplitude decreased, until arrest usually occurred in systole in from 9 to 12 minutes, although a few hearts stopped in diastole. Hearts which had been arrested by other means could often be temporarily revived by isotonic sodium chloride (cf. Rogers, 1905). Isotonic glucose (0.45 molar) gave somewhat variable results. In ten hearts a markedly decreased frequency and a gradually increased tone were followed by arrest in systole or near systole within 5 minutes. In three hearts the frequency, the amplitude, and the tone decreased, and arrest occurred in diastole. In one heart the frequency and amplitude decreased, but the tone remained unchanged up to arrest in diastole. Similarly, mixtures of isotonic glucose and sodium chloride gave equivocal results as far as arrest was concerned. However, the typical sodium chloride effect of initially increased frequency and amplitude occurred in mixtures containing 25 per cent of the normal sodium chloride content or more, while a decreased frequency and amplitude occurred in mixtures containing less than this proportion.

By decreasing the molarity of van Harreveld's solution one-half, without altering the ionic ratios (solution 19), marked increases in frequency and amplitude with a slight increase in tone occurred, followed by gradual fatigue. Amplitude decreased faster than frequency, and the beat continued for as long as 20 minutes, when arrest in systole occurred. Recovery in van Harreveld's, however, was incomplete, indicating a certain amount of irreversible injury. Doubling the molarity of van Harreveld's solutions (solution 20) without changing ionic ratios caused quick arrest in diastole, with good recovery in van Harreveld's within a few minutes.

The other solutions tested showed the following qualitative results. The effects produced on the heart were in all cases roughly proportional to the amount of change in cation concentration, the greater effects being produced by the larger changes. Excess of sodium or deficiency of potassium (solutions 4, 14, 21, and 22) caused increased frequency and slightly increased amplitude; excess of potassium or deficiency of sodium (solutions 5, 7, 9, 10, 13, 29–32) caused decreased frequency and increased amplitude; excess of calcium (solutions 8–10, 12, 13, 17, 27–30) and magnesium (solutions 33 and 34) caused decreased frequency and amplitude; a deficiency of calcium (solutions 11, 14, 18, 22, and 23) caused increased frequency and amplitude.

Quantitatively, the most important ionic ratios are those of potassium and of calcium to sodium. As indicated earlier, the magnesium content is of little importance unless a large excess is present (284 and 650 per cent in solutions 33 and 34). Decreasing potassium by 50 per cent (solution 21) caused only a slightly increased rate; increasing it 17 per cent (solution 5) caused no effect. Small changes in calcium, however, caused notice-

able effects (7 per cent decrease in solution 11, 21 per cent decrease in solution 23, and 9 per cent increase in solution 12). Table 3 gives the qualitative effects of increasing the number of potassium and calcium ions per 100 sodium ions in those solutions in which the magnesium and the sodium ions were not significantly changed. Expressed on the basis of 100 sodium ions, the normal content is 2.63 potassium ions and 6.58 calcium ions. The former can probably change by 0.75 (nearly 30 per cent); but the latter by only about 0.25 (about 4 per cent) without causing changes in the heart beat for several minutes. The limits of calcium are, therefore, very narrow; of potassium considerably wider; of sodium and magnesium still wider. The large increases in potassium (270 and 350 per cent in solutions 32 and 31) caused a marked decrease in frequency and a significant increase in amplitude, the latter being followed in from 2 to 5 minutes by a gradually de-

TABLE 3

THE EFFECTS OF INCREASING K AND Ca RATIOS (RELATIVE TO Na = 100), IN SOLUTIONS USED ON THE CRAYFISH HEART

Solution	K	Percentage of Change	Ca	Percentage of Change	Effects on Heart
2	0	.....	6.37	.....	Increased rate and tone on fresh hearts
21	1.31	−50	6.55	.....	Slightly increased rate on fresh heart
v.H.	2.63	.....	6.54	.....	"Normal"
1	2.63	.....	0	.....	Increased rate; systolic arrest
23	2.64	.....	4.94	−25	Increased rate
11	2.62	.....	6.04	−8	Increased rate
18	2.63	.....	6.23	−5	Slightly increased rate
v.H.	2.63	.....	6.58	.....	"Normal"
12	2.66	.....	7.24	+10	Decreased rate
17	2.62	.....	7.92	+20	Decreased rate
27	2.67	.....	10.1	+53	Decreased rate
8	2.62	.....	12.2	+82	Decreased rate and amplitude
5	3.09	+17	6.54	.....	No real effect
7	4.85	+84	6.56	.....	Decreased rate
32	10.5	+300	6.55	.....	Decreased rate and increased amplitude
31	13.2	+400	6.58	.....	Decreased rate; increased tone and amplitude

creasing amplitude and increasing tone. Feeble and slow contractions, however, might continue up to 10 minutes before arrest occurred in systole. In view of the fact that excess potassium "narcotizes" the peripheral nerves of crustaceans (Cowan, 1934), the continued beating of the crayfish heart in solutions 30, 31, and 32 may indicate at least temporary independence of nervous control.

SUMMARY

1. Van Harreveld's solution is far more satisfactory for perfusing the crayfish heart (*Cambarus clarkii*) than Lindeman's or double Ringer's solution. Hearts have continued beating for as long as 20 hours in van Harreveld's.

2. Moderate changes in pH (from 6.4 to 8.4) and slight changes in osmotic pressure do not affect the heart for several hours. Halving the osmotic pressure causes increased frequency and amplitude; doubling it causes quick arrest in diastole.

3. The heart beat is primarily controlled by the sodium ion, which in the absence of other cations causes too high a frequency and amplitude leading to quick fatigue.

4. Potassium, calcium, and magnesium retard the frequency, and a proper balance between them and sodium is necessary for continued beating.

5. The calcium ion is by far the most important antagonist to sodium. Its optimum concentration can be changed by only about 5 per cent without affecting the heart.

6. Increased frequency is caused by excess of sodium or by a lack of potassium, calcium, or magnesium, and decreased frequency by a lack of sodium or by an excess of potassium, calcium, or magnesium.

7. Increased amplitude is caused by excess of sodium or potassium, and decreased amplitude by excess of calcium or magnesium.

8. Arrest in systole is caused by isotonic NaCl, KCl, or $NaHCO_3$, and arrest in diastole by isotonic $CaCl_2$ or $MgCl_2$.

9. Isotonic glucose is more harmful than isotonic NaCl, usually causing arrest in systole or near systole, although a few hearts were arrested in diastole. Mixtures of isotonic glucose and sodium chloride containing 25 per cent or more of the normal NaCl content produced the typical sodium chloride effect of initially increased frequency and amplitude. Mixtures containing less than 25 per cent of the normal NaCl content caused decreased frequency and amplitude, the effect typical of pure glucose solutions.

10. The perfusion solution for the crayfish heart must correspond closely in its inorganic content with the blood serum of the crayfish, if normal behavior of the heart is to be maintained. Simplified solutions may profoundly alter the heart beat, even though it may continue for several hours.

11. The heart may adapt itself to an incorrect solution and continue beating for a considerable time, but its reactions to changes in the perfusing solution may be quite different from those secured following perfusion by a different initial solution.

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SEROLOGICAL INVESTIGATION OF RODENT RELATIONSHIPS

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IT WAS the purpose of this investigation to study the precipitin reaction with anti-rodent rabbit sera, to determine the serological relationships of a series of rodents, and to test the applicability of the reciprocal reaction when the antibody-producing agent (rabbit) belongs to an order of mammals (Lagomorpha) presumably closely related to the order (Rodentia) which supplies the antigen.

Nuttall (1904) employed the precipitin reaction in extensive experiments on the serological relationships of animals. He concluded that "the degree and rate of blood reaction appear to offer an index of the degree of blood relationship." The results of his tests were in general accord with the accepted zoölogical classification.

Nuttall and subsequent investigators studied the serological relationships of representatives of practically all the mammalian orders except the rodents, which were omitted mainly because of the technical difficulty of obtaining suitable anti-rodent sera. The use of fowl as antibody-producing agents results in cloudy antisera (Nuttall, 1904; Boyden, 1926; Hicks and Little, 1931). Earlier investigations (Nuttall, 1904; Boyden, 1926) seemed to indicate that rabbits are unsuitable for developing rodent antisera of sufficient potency for use in determining serological relationships, possibly because of the close relationship between the two. However, Uhlenhuth (1905) was successful in producing anti-hare serum in rabbits, and Hicks and Little (1931) were able to produce antibodies in rabbits against rats and mice in 25 per cent of their attempts. Erhardt (1931), in an extensive review, cited very few investigations with anti-rodent precipitins, and these accomplished little beyond differentiating between the blood sera of rats and mice.

MATERIALS AND METHODS

Antigens used in this work were the blood sera from various rodents. Some sera were obtained from blood samples collected in the field, others were from laboratory stock. In the latter instances blood was obtained by cardiac puncture under ether anesthesia after the manner of Hicks and Little (1931). The blood of the smaller animals was pooled. The blood was centrifuged, and the supernatant serum was pipetted off and stored in the refrigerator in sterile sealed ampules. Aseptic precautions were observed throughout. The blood collected under field conditions was centrifuged, and the supernatant serum removed and passed through sterile Seitz filters before storage.

Standard 1 per cent antigen dilutions were prepared from the whole serum by using sterile 0.85 per cent NaCl buffered to neutrality with Sørenson's phosphate buffers as recommended by Evans (1922). These standard solutions were passed through sterile Seitz filters and stored in small sealed ampules.

Normal, healthy rabbits were used as precipitin-producing agents. Each animal was injected intravenously with 0.2 cc. of whole serum daily for six or seven days. After a rest period of one week another similar series of daily injections was given. Eight to ten days after the last injection, 50–60 cc. of blood were removed by cardiac puncture,

under ether anesthesia. The blood was defibrinated and centrifuged, and the supernatant antiserum was stored in sterile-sealed ampules in the refrigerator.

Occasional attempts to increase the titer of an antiserum by giving a third series of injections were usually unsuccessful in increasing the homologous titer, though the heterologous titers might be altered. This agrees with the findings of Wolfe (1935, 1936), whose results on the effect of injection methods in altering specificity prompted us to give five of the rabbits a third series of six daily injections starting one to two days after bleeding. Eight to ten days after the last injection 50–60 cc. of blood were again removed, and the antiserum was obtained and stored for comparison with the antiserum from the first bleeding of the same rabbits.

In performing the precipitin tests, the "ring-test" method was used because it was found to be much more constant in results than the original flocculation test (unpublished data; cf. also Boyden, 1926).

Preliminary tests were first made on each antiserum to determine its homologous titer. For this purpose an arithmetically progressing series of dilutions was prepared from the standard 1 per cent antigen with sterile buffered saline. In small sterile test tubes 0.2 cc. of antigen dilution was overlaid on 0.1 cc. of homologous antiserum. The tubes were incubated in a water bath for 1 hour at $37^{\circ}.5$ C $\pm$ $0^{\circ}.5$. The highest antigen dilution in which a white ring was noticeable at the antigen-antiserum interface was considered the homologous titer of the antiserum.

In performing the actual experimental tests, standard solutions of the homologous and various heterologous sera were diluted according to one or another of a set of arithmetic progressions. These progressions were so arranged that each homologous series usually contained the same number of dilutions regardless of the homologous titer of any antiserum. Thus, if the preliminary titer was about 1:25,000, a dilution series was used which increased by steps of 1:4,000; if the preliminary titer was about 1:20,000, the dilution series increased by steps of 1:3,000. A series increasing by steps of 1:2,500 was employed when the preliminary titer was less than 1:15,000. This method should facilitate comparison between antisera of different titers.

The tests were set up and incubated as described above. The end-point titers of the heterologous antigens were recorded and compared with the homologous end point. As a control, a tube containing 0.1 cc. of antiserum overlaid with 0.2 cc. of buffered saline was incubated with the others. The control was always clear. The possible observational error in reading the end-point titer was found to be $\pm$ one tube within the limits of the dilutions used in this study.

EXPERIMENTAL DATA AND RESULTS

Production of rodent antisera.—Table 1 records the twenty-four antisera produced, the number of injections, the total amount of antigen injected, and the homologous titer of each. There is considerable variability in antibody production; some antisera react strongly with the homologous antigen, others not at all. Furthermore, this variability in homologous reaction is not correlated with the amount of antigen injected or with the injection method, but appears to be an individual reaction of the rabbit. For example, Rabbits 12, 14, and 21 show little change in *homologous* titer upon repeated injections. Rabbits 17, 18, and 23*a*, treated identically, differ greatly in homologous titer.

Effect of antigen aging upon the precipitin test.—Since some of the sera had been kept 3 years, it was advisable to test the effect of storage. It was found that standard anti-

gens prepared from serum 3 years old and those from freshly prepared serum of the same species reacted to the same degree, within the limits of the observational error, with any one antiserum. The same results were observed with standard solutions prepared 6 months apart from the same serum sample. This constancy of reaction of different sera

TABLE 1

PRODUCTION OF RODENT IMMUNE RABBIT SERA

Rabbit No.	Antiserum For	No. of Injections	Total Injection (Cc.)	Homologous Titer
Q*	Deer mouse	11	5.5	1:30,000
T	Deer mouse	21	6.0	1:1,000
U	Porcupine	17	3.2	1:18,000
V	Wood rat	20	4.1	1:15,000
1	Porcupine	32	6.3	1:21,000
2	Woodchuck	25	4.9	1:20,000
3	Pocket gopher	13	2.6	0
4	Wood rat	13	2.6	1:24,000
5	Woodchuck	13	2.6	1:18,000
6†	Deer mouse	14	2.8	1:28,000
7	Porcupine	14	2.8	1:18,000
8‡	Pocket gopher	12	2.4	0
9‡	Woodchuck	12	2.4	1:15,000
10	Cotton rat	12	2.4	1:28,000
12*a*	Muskrat	12	2.4	1:7,500
12*b*	Muskrat	18	3.6	1:10,000
13	Roof rat	12	2.4	1:10,000
14*a*	Deer mouse	12	2.4	1:28,000
14*b*	Deer mouse	18	3.6	1:28,000
16	Woodchuck	12	2.4	1:15,000
17	Porcupine	12	2.4	1:15,000
18	Wood rat	12	2.4	1:32,000
19	Roof rat	9	1.5	1:15,000
20*a*	Muskrat	12	2.4	1:1,000
20*b*	Muskrat	18	3.6	1:7,500
21*a*	Woodchuck	12	2.4	1:18,000
21*b*	Woodchuck	18	3.6	1:18,000
23*a*	Porcupine	12	2.4	0?
23*b*	Porcupine	18	3.6	0?

* Injected with a mixture of powdered egg albumin solution and deer-mouse serum in the hope that the heightened reaction resulting from the development of a high-titered anti-egg albumin serum would induce an increase in the titer developed against the deer-mouse serum (cf. Zinsser, 1931, p. 474).

† Injected with citrated plasma. The reactions of this antiserum were quite comparable with the reactions obtained with antisera from serum-immune rabbits.

‡ Injected with 50 mg. of vitamin C (crystalline Merck), dissolved in 0.85 per cent NaCl, just prior to each daily injection of serum (Jusatz, 1936). No increase in antibody production was noted.

from the same species was apparent throughout the whole investigation. Barring pathological conditions, blood sera of varying ages from the same species can be expected to react to the same degree with a given antiserum.

Effect of aging of antisera upon specificity.—Three antisera (Rabbits U, V, and 2) which had been stored for 60, 80, and 45 weeks, respectively, were tested for comparison with the reactions previously obtained with the same antisera after storage 2–7 weeks. There was a marked increase in specificity as manifested by a retention of the original homologous titer and reduction in the heterologous titers; the weaker heterologous reac-

tions disappeared entirely. Similar observations were reported by Wolfe (1933). Retention of a homologous reaction is no indication of retention of relative specificity. From the foregoing results it is evident that, in order to test the relationships of various heterologous sera to a homologous serum, the tests should be performed with antisera of about the same age.

Effect of variation in total nitrogen content of antigens.—In view of the fact that antigens for the precipitin test are sometimes diluted in terms of their nitrogen content, it seemed desirable to determine the nitrogen content of the antigens employed in the present work. Total nitrogen determinations were made by the Pregl (1930) micro-Kjeldahl method on most of the 1 per cent standard solutions from seven of the antigens used.[1] With one exception the total nitrogen content did not vary widely from species to species. Table 2

TABLE 2

VARIATION IN TOTAL NITROGEN OF 1 PER CENT STANDARD ANTIGEN SOLUTIONS PREPARED FOR EACH SPECIES AT DIFFERENT TIMES

All tests were performed in duplicate. The values are the averages of such duplicate tests and represent mg. of nitrogen per 5 cc. of 1 per cent solution.

Species	No. of Solutions Tested	Highest Determination	Lowest Determination	Difference	Average Determination
Deer mouse	6	0.360	0.220	0.140	0.285
Pocket gopher	3	0.275	0.155	0.120	0.197
Wood rat	1*	0.597	0.540	0.057	0.568
Woodchuck	2	0.234	0.199	0.035	0.217
Porcupine	1*	0.195	0.192	0.003	0.193
Cotton rat	1	.....	.....	.....	0.190
Muskrat	1	.....	.....	.....	0.152

* Two determinations made on different days on portions of the same standard serum solution.

gives the average reading for each species and also records for each species the variation in nitrogen content of standard solutions prepared at different times. The standard solutions were prepared from pooled sera in the cases of deer mouse, pocket gopher, and cotton rat. The muskrat, porcupine, and wood rat are each represented in the table by the serum of a single individual, though serum from more than one individual of the latter two species was used in the precipitin tests. The two standard solutions of the woodchuck were prepared from the sera of two different individuals, respectively.

It will be noted from Table 2 that, with the exception of the wood-rat serum, the differences between species are not greater than the differences which may arise between determinations of different samples from the same species. Thus, the difference between the averages of deer mouse and muskrat is 0.133 mg., all the other interspecific differences being less, except for the wood rat. It will be seen from the fifth column of the table that variations between serum samples prepared at different times from a single species may easily equal or surpass such interspecific differences. These variations within a species may be due to variation in the accuracy of pipetting in the preparation of stand-

[1] We wish to express our appreciation to Miss Thera Visscher, technical assistant in the Department of Zoölogy, who performed these tests.

ard solutions, to error in the micro-Kjeldahl test, to possible fluctuations in concentration of serum proteins, to differences in non-protein nitrogen due to the varying metabolic states of the animals at the moment of bleeding (particularly when pooled sera are not used), or to a variety of other factors.

The reason for the much higher nitrogen content of the wood-rat serum is not evident. It is of interest to note that this greater nitrogen content did not result in the production of antisera of consistently higher titer than was elicited by antigens of lower nitrogen content (Table 1). No nitrogen determinations were made on the samples of whole serum used for the inoculation of rabbits, but there is no reason to doubt that their nitrogen concentrations were proportional to those of the dilute solutions on which such determinations were made.

TABLE 3

PRECIPITIN REACTIONS OF CLOSELY RELATED RODENT SERA WITH ANTISERA OF DIFFERENT SPECIFICITIES

Rabbit	Homologous Titer	Deer Mouse	Wood Rat	Cotton Rat
7	1:18,000	1:9,000	1:12,000	1:6,000
12*a*	1:7,500	1:1,000	0	0
12*b*	1:10,000	1:10,000	1:10,000	1:7,500
17	1:15,000	1:1,000	1:3,000	1:1,000
19	1:15,000	1:3,000	1:6,000	1:1,000
20	1:7,500	0?	1:1,000	1:1,000
21	1:18,000	1:3,000	1:3,000	1:3,000

Rabbit	Homologous Titer	Gray Squirrel	Woodchuck
6	1:28,000	1:1,000	1:1,000
7	1:18,000	1:1,000	1:6,000
10	1:28,000	1:4,000	1:1,000
12*b*	1:10,000	1:1,000	1:1,000
14*a*	1:28,000	1:1,000	1:4,000
14*b*	1:28,000	1:4,000	1:8,000
18	1:32,000	0	1:4,000

The greater nitrogen content of the wood-rat standard solutions did not appear to modify the values obtained in the precipitin tests. From the upper portion of Table 3 it is evident that with a variety of antisera the three closely related forms—deer mouse, wood rat, and cotton rat—gave reactions of closely similar magnitude. The standard solution of wood-rat serum included in Table 2 and an additional one were used for the tests included in Table 3; the two reacted similarly. Most of the differences in end point shown in Table 3 are within the observational error of $\pm$ one tube (see above). For example, in but two cases was there a difference of two tubes between the end points with wood-rat antigen (N=0.593 mg.) and those with cotton-rat antigen (N=0.190 mg.).

It seems evident that, within the limits of the series of forms here employed, variation between species in total nitrogen content of normal serum does not form a significant variable as compared with the magnitude of the other variables involved.

Influence of increased antigen injections upon specificity.—The precipitin reactions of the antisera from two different bleedings of five rabbits (see Table 1) were compared.

One (Rabbit 23) failed to produce any measurable titer at either bleeding; another (Rabbit 21) gave identical reactions with the antisera from both bleedings. Of the remaining three (see Table 4), Rabbits 12 and 14 revealed a loss in specificity, evidenced by increase in heterologous titers with tendency to retain homologous titers. This agrees with the results of Wolfe (1935, 1936, 1939) and corroborates his conclusion that, when comparable results are desired, as in determining zoölogical relationships, a uniform injection procedure should be maintained. The loss in specificity was less apparent in the two bleedings from Rabbit 20, since the first bleeding yielded a very weak antiserum. It is interesting to note that the antiserum from the second bleeding of Rabbit 20 had the same order of specificity as the antiserum obtained from the first bleeding of Rabbit 12. This seems to indicate that rabbits may differ in speed of antibody production as well as in strength of antibody production.

Tests of rodent relationships by means of the precipitin reaction.—The taxonomic relationships of the rodents studied in this work[2] are given by Miller (1923) as follows:

ORDER RODENTIA

I. Superfamily Sciuroidae
  A. Family Sciuridae
    1. Subfamily Sciurinae
      a) *Marmota monax* (woodchuck)
      b) *Sciurus carolinensis* (gray squirrel)
  B. Family Geomyidae
      Geomys bursarius (pocket gopher)
II. Superfamily Muroidae
  A. Family Cricetidae
    1. Subfamily Cricetinae
      a) *Peromyscus gossypinus* (deer mouse)
      b) *Sigmodon hispidus* (cotton rat)
      c) *Neotoma albigula* (wood rat)
    2. Subfamily Microtinae
      Ondatra zibethica (muskrat)
  B. Family Muridae
      Rattus rattus alexandrinus (roof rat)
III. Superfamily Hystricoidae
  A. Family Erethizontidae
      Erethizon dorsatum (porcupine)

The rabbits and hares were formerly regarded as constituting a suborder within the order Rodentia. Mammalogists now include them in a separate order (Lagomorpha), which is usually placed immediately following order Rodentia in the classification (for status of the group see Gidley, 1912). The exact interrelationship of the two orders has not been determined. Nevertheless, it seems probable that the rodents are more closely related to the lagomorphs than are the other orders of mammals, although there is not unanimity of opinion on this point.

The results obtained with nineteen antisera against seven of the nine rodents studied

[2] We are indebted for material to Dr. Lee R. Dice and Dr. Philip Blossom, of the Laboratory of Vertebrate Genetics, University of Michigan; to Dr. Robert B. Aiken, Mr. Herbert Daigneault, Mr. Robert Fyfe, and Mr. Avery King.

are shown in Table 4. Three attempts to produce anti–pocket-gopher serum failed; limited supply of serum prevented attempts to produce anti-squirrel serum.

Because of possible effects on specificity, injection methods were kept constant (see Table 1), and tests were performed with antisera 7–12 weeks old. Even under these conditions antisera of different specificities were produced. For example, antisera from

TABLE 4

RESULTS (IN PERCENTAGE OF HOMOLOGOUS REACTION) OF PRECIPITIN TESTS EMPLOYING VARIOUS RODENT ANTISERA TESTED WITH A SERIES OF RODENT SERA

RABBIT	ANTISERUM	ANTIGENS								
		Deer Mouse	Wood Rat	Cotton Rat	Roof Rat	Muskrat	Porcupine	Woodchuck	Gray Squirrel	Pocket Gopher
Q	Deer mouse	100	83	.....	.....	.....	50	.....	.....	33
6*	Deer mouse	100	71	71	43	14	0	4	4	4
14a*	Deer mouse	100	71	71	43	29	4	14	4	14
14b	Deer mouse	100	100	100	57	43	14	29	14	14
V	Wood rat	83	100	.....	.....	.....	50	.....	.....	.....
4*	Wood rat	67	100	.....	.....	.....	4	0	.....	4
18*	Wood rat	75	100	75	50	38	38	13	0	3
10*	Cotton rat	71	71	100	29	29	14	4	14	4
13	Roof rat	0	0	0	100	0	0	.....	.....	0
19	Roof rat	20	40	7	100	7	.....	0	0	0
12a	Muskrat	13	0	0	0	100	0	0	0	0
12b	Muskrat	100	100	75	100	100	10	10	10	0
20a	Muskrat	0	0	0	.....	100	.....	.....	.....	.....
20b	Muskrat	0?	13	13	0	100	0	0	0	0
1*	Porcupine	14	71	.....	.....	.....	100	29	.....	5
7*	Porcupine	50	67	33	50	17	100	33	6	0
17	Porcupine	7	20	7	0	7	100	0	0	0
2	Woodchuck	5	.....	.....	.....	.....	50	100	.....	0
5	Woodchuck	0	0	0	0	0	0	100	83	0
16	Woodchuck	0	0	0	0	0	7	100	100	0
21*	Woodchuck	17	33	17	0	6	33	100	100	6

* Antisera used in Table 5.

a Antisera from rabbits after two injection series.

b Antisera from the same rabbits after a third injection series.

Rabbits 14*a* and 10 reacted with all the heterologous sera, while that from Rabbit 13 reacted only with the homologous antigen; various intermediate degrees of specificity occurred.

Despite these differences of specificity among the antisera, the results indicate a serological relationship among the rodents. With few exceptions the relative positions of the heterologous sera to the homologous serum are the same with antisera against the same species even though the actual quantitative results differ widely. The exceptions involve apparent reversals in the relative positions of some of the weaker heterologous re-

actions. It may be noted that most of these reversals include porcupine serum or antiserum, though the significance of that fact is not yet apparent.

Analysis of the serological relationships shows striking agreement with the accepted taxonomic relationships. With deer-mouse antisera (Table 4) the largest heterologous reactions occurred with wood-rat and cotton-rat sera, both of which are regarded as different genera within the same subfamily as the deer mouse. Next in magnitude were the reactions with roof rat and muskrat, which are in the same superfamily as the deer mouse. The weakest reactions involved rodents morphologically more distantly related to the deer mouse. Reactions with wood-rat and cotton-rat antisera revealed the same relationships; deer-mouse, wood-rat, and cotton-rat sera reacted most strongly, followed by roof-rat and muskrat reactions. Two roof-rat antisera (Rabbits 13 and 19) differed widely in specificity; one reacted only with the homologous serum, the other with the four more closely related heterologous sera. Agreement between the serological and morphological relationships was still apparent, since the heterologous reactions included only members within the same superfamily as the roof rat. Essentially similar results were obtained with anti-muskrat sera.

The results with anti-porcupine sera are of doubtful significance in determining serological relationships because of the shifts already noted in relative position of the heterologous reactions from antiserum to antiserum.

With the woodchuck antisera the strongest heterologous reaction occurred with gray-squirrel serum; these two forms are regarded as related genera within the same subfamily. With woodchuck and porcupine antisera there is one marked difference between the serological results and the accepted classification. With woodchuck antisera, porcupine serum reacted more strongly than did pocket-gopher serum; with anti-porcupine sera the woodchuck reacted more strongly than did the pocket gopher. This points to a closer relationship between the woodchuck and the porcupine than between either and the pocket gopher. Yet, the woodchuck and pocket gopher are regarded as members of the same superfamily, while the porcupine is placed in a distantly related superfamily. Because of the doubtful value of the porcupine reactions, however, definite conclusions must await further investigation.

Application of the reciprocal reaction.—Boyden (1926, 1934) formulated the principle of reciprocal relationships in serological investigations: "The percent values of the relationships between any two species A and B should be of the same order of magnitude, whether antiserum A is tested with antigen B or antiserum B is tested with antigen A." However, he found that, with antisera built up in rabbits, the proper relationship of rodents to other animals could not be determined, owing to an inhibition of rabbit response to rodent antigens. This lack of reaction was not apparent with antisera produced in fowl. Therefore he stated, "The principle of reciprocal relationships holds only when the antisera are produced by animals which are about equally distant from the species tested." Reciprocal testing with anti-rodent rabbit sera is in accord with this reasoning, since any two rodents, as contrasted with a non-rodent, are about equally removed from the rabbit.

Owing to the extreme differences in specificity, it is obvious that positive reciprocal reactions could not be obtained if all the antisera were compared. Table 5 records the reciprocal tests for five rodents obtained with eight antisera which appeared to have the same degree of specificity (checked with an asterisk in Table 4). Of ten reciprocals, six were definitely positive within the possible observational error (± one tube), one (wood-

chuck vs. wood rat) was doubtful, and three involving porcupine reactions were doubtful or negative. This is further indication that the porcupine reactions are aberrant and of doubtful value for determining serological relationships. The results indicate that the reciprocal test is valuable in checking relationships among the rodents *if antisera are compared which have the same order of specificity.*

Another possible method of checking the reliability of the precipitin reaction is suggested by the assumption that antigens which are closely related serologically should react to about the same degree with any antiserum regardless of its specificity (Wolfe, 1933). To illustrate this point, we may cite results with deer mouse, wood rat, and cotton rat, and with squirrel and woodchuck. The tests show that the first three are closely

TABLE 5

RECIPROCAL REACTIONS (IN PERCENTAGE OF HOMOLOGOUS REACTION) WITH ANTISERA OF THE SAME ORDER OF SPECIFICITY

Homologous Antigen	Heterologous Antigen	Percentage Reaction	Homologous Antigen	Heterologous Antigen	Percentage Reaction	Diff.
Deer mouse	Wood rat	71*	Wood rat	Deer mouse	71*	0
Deer mouse	Cotton rat	71*	Cotton rat	Deer mouse	71	0
Deer mouse	Porcupine	4	Porcupine	Deer mouse	32*	28x
Deer mouse	Woodchuck	9*	Woodchuck	Deer mouse	17	8
Wood rat	Cotton rat	75	Cotton rat	Wood rat	71	4
Wood rat	Porcupine	21*	Porcupine	Wood rat	69*	48x
Wood rat	Woodchuck	13	Woodchuck	Wood rat	33	20?
Cotton rat	Porcupine	14	Porcupine	Cotton rat	33	19?
Cotton rat	Woodchuck	4	Woodchuck	Cotton rat	17	13
Porcupine	Woodchuck	31*	Woodchuck	Porcupine	33	2

* Average of results from two antisera.
x Negative reciprocal reaction.
? Doubtful reciprocal reaction.

interrelated, as are the last two. Table 3 records the reactions of these animals with antisera of varying specificities. The closely related rodents reacted to the same degree, within the possible observational error, in ten out of fourteen cases. Of the four negative reactions, none differed by more than two dilution tubes.

DISCUSSION

Nuttall (1904) was of the opinion that a powerful anti-rodent serum, if obtained, would react generally with all rodents, since the lack of reaction of rodent sera with non-rodent antisera suggested that the rodents were a closely related serological group. But subsequent results have not confirmed this opinion. Hicks and Little (1931) found serological differences among four species within the genus Mus. Avrech and Kalabuchov (1937) reported differentiation between species and even subspecies within the mouse genus Apodemus. Wolfe (1939) was able to distinguish between genera within the family Sciuridae. In the present work closely related genera within the same subfamily were

differentiated. It is possible that species within a genus might have been distinguished with some of the very specific antisera if the proper antigens had been available.

Differentiation between rodents is to be expected when rabbits are the antibody-forming agents. The rodents and rabbits are presumably closely related and possibly possess some serum proteins in common. Since antibodies are formed against foreign proteins, it can be understood why rabbits should produce specific anti-rodent sera. Landsteiner (1936, p. 13) observed:

> Apparently, the degree of relationship between the animal species furnishing the antigen and that used for immunization has considerable influence on the results . . . . rabbit immune sera are very suitable for revealing dissimilarities in the proteins of other rodents, while in the case of birds, if rabbit sera are employed, the lesser differences will be hidden by structures common to bird proteins.

Supportive evidence was given by Hicks and Little (1931), who were unable to distinguish between rat and mouse sera by use of pigeon-immune sera, though they succeeded with rabbit-immune sera.

Injection methods and the age of antisera were shown to affect their specificity. It is important that these factors be kept constant where comparable results are desired. Even when these conditions are controlled, however, antisera of widely differing specificities are produced. Such differing specificities may be due to intrinsic differences in the rabbits producing the antisera, or to some property of the injected serum manifested by apparent individual variation of response among the rabbits. Relative to this second possibility it may be noted that Heidelberger and Kendall (1935) gave successive courses of injections of purified egg albumin to rabbits and noted progressive changes "believed to consist in the formation of more and more antibody capable of reacting with a larger number of chemically different groupings in the antigen molecule." Hooker (1937) postulated the existence of multiple antigenic determinants, possessing a possible diversity of antigenicity, upon the same protein molecule. Although blood serum is composed of a complex mixture of proteins rather than of a single protein, these views suggest a possible explanation for the observed differences in the reactivity of the antisera.

In such an antigenic complex as the blood serum, the number of proteins (and/or protein radicals) common to a series of rodents would presumably be in direct ratio to the degree of relationship of the rodents to one another. Closely related rodents would have more proteins (and/or radicals) in common than would distantly related rodents. Individual variation of rabbits in response to the lesser constituents of the serum might loom large in affecting the precipitin reactions subsequently given by an antiserum to distantly related antigens. Such variations might lead to discrepancies and even reversals from antiserum to antiserum. Varying amounts and injection schedules of antigen might also be expected to produce similar variations in the resultant antibody formation against these minor substances.

In spite of these variations in antisera, the results indicated a quantitative serological relationship among the rodents studied. In most cases the relative positions were the same from antiserum to antiserum. A strictly quantitative relationship among the rodents could not be determined, however, because of the extreme differences in antiserum specificity.

Boyden's application of the reciprocal reaction (1934) resulted in a three-dimensioned phylogenetic tree showing the quantitative relationships of five mammalian sera to one

another. The animals furnishing the sera were rather distantly related to one another and also to the antibody-producing rabbits; the quantitative relationships obtained were of a general nature. Wolfe (1933, 1935) could not obtain constant reciprocal reactions with a series of carnivore sera. In the present work it was apparent that the reciprocal reactions would not check if all the antisera were considered. This affords further evidence that individual differences among antisera are conspicuous in work with closely related antigens.

Excellent agreement in reciprocal reactions was obtained, however, when antisera of about the same order of reactivity were tested. Under these conditions reciprocal reactions established the relative relationships of the rodents tested, though the actual percentages obtained were rather a measure of the specificity of the antisera than a strictly quantitative measure of the relationships of the antigens. Even so, since it is found that antisera of like specificity give measurably comparable reciprocal reactions, the reciprocal test is valuable in checking the relationships of even closely related groups. The problem of establishing a truly quantitative phylogeny, as distinguished from a phylogeny of relative position, however, is yet to be solved, at least for such closely related animals as the rodents here employed.

SUMMARY

1. Rodent-immune rabbit sera were obtained by intravenous injections of small daily doses of serum over an extended period of time.

2. The age of an antigen had no effect upon the precipitin reaction.

3. Increased antiserum age resulted in increased specificity as manifested by reduction in heterologous titer, while the homologous titer remained nearly constant.

4. Increased number of antigen injections resulted in decrease in antiserum specificity as manifested by increase in heterologous titer, while the homologous titer remained nearly constant.

5. Within the limits of the series of forms here employed, variation between species in total nitrogen content of normal serum does not form a significant variable.

6. Even when the foregoing conditions were kept constant, antisera of different specificities were produced. Possible explanations are discussed. Such individual differences in specificity appear to form a more significant source of error in investigations involving closely related forms than in studies of more distantly related animals.

7. Positive reciprocal results were obtained if antisera were compared which were of the same order of specificity.

8. Serologically closely related antigens were found to react to the same degree with any antiserum regardless of its specificity.

9. Serological relationships of a series of rodents showed striking agreement with the morphological classification with one possible exception. Rodents of different superfamilies were separated very readily; families and subfamilies within the same superfamily were distinguished from one another; in many instances it was possible to differentiate closely related genera within the same subfamily.

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MODIFICATION OF THE SOCIAL ORDER IN FLOCKS OF HENS BY THE INJECTION OF TESTOSTERONE PROPIONATE[1]

(One figure)

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MODERN scientific recognition of the existence of a definite social organization within small flocks of birds dates from the work of Schjelderup-Ebbe (1922) on the so-called "peck-order" in the common domestic fowl (*Gallus domesticus*). He has also observed the phenomenon which he calls "despotism" in a large number of birds, and he summarized his work on the subject in 1935. Schjelderup-Ebbe's original description for chickens[2] has been essentially verified, among others, by Masure and Allee (1934) and by Murchison in his 1935 series of papers.

This social organization is based primarily on the outcome of an initial pair-contact, or a series of such contacts, in which one bird loses a fight or submits passively without fighting. Thereafter, when these two fowls meet, the one which has acquired the peck-right (that is, the right to peck without being pecked in return) exercises it frequently. The subservient bird of an original pair often avoids close contact with her superior and, in event of a revolt, usually does not again fight back vigorously Revolts occur rarely among hens, and successful revolts that result in a change in social status are still more rare.

Factors which make for dominance between members of the same sex and species of birds have been reported to include the following (see Schjelderup-Ebbe, 1935; Shoemaker, 1939):

1. Other things being equal, the stronger bird usually wins.
2. One bird acts as though intimidated by the appearance of a strange and apparently unfrightened individual and so gives way without fighting.
3. Both birds act as though frightened, but one recovers more rapidly and so wins the contact reaction.
4. An individual which is normally aggressive and victorious may be temporarily out of condition (tired or ill or molting severely) when it meets a newcomer, and so loses when it might, from other relations, be expected to win.
5. Mature hens usually dominate immature chickens.
6. The location of the first contact is important; birds, like many mammals, fight better in their home territory.
7. Even in territory strange to both members of a contact pair a bird wins more readily if surrounded by others with which it has associated.

[1] This work has been supported in part by a grant from the Committee on Research in Endocrinology of the National Research Council. We are indebted to Professors C. R. Moore and F. C. Koch and to Drs. L. V. Domm and Walter Hoskins for advice concerning the application of hormones to our particular problem and to certain of them for reading this manuscript.

[2] The *Standard Dictionary* defines "chicken" as "the young of the common fowl; loosely, a fowl of any age. . . . ." The word is used here in this looser sense.

8. Birds with young fight more fiercely than they would at others times. With some birds—canaries, for example—intensity of fighting varies with the different phases of the reproductive cycle.

Two main types of social order have been described for birds. Among chickens, for example, once social dominance is established, it tends to be maintained unless there is a decided revolt. The dominant birds seem to have an absolute peck-right over their subordinates. With several other species—of which pigeons, doves, and shell parakeets may be mentioned—the result of any given pair-contact can seldom be predicted with certainty, even though, in the long run, one of the pair wins more frequently than does the other. Masure and Allee (1934) called this a "peck-dominance," in contrast with the peck-right found among chickens.

It is readily seen that with animals that have an almost absolute peck-right any reverse pecks which may be observed are much more significant than they would be if the society were organized about peck-dominance. Hence, for the present studies the use of the common domestic fowl was indicated. Also, since cocks have a more unstable peck-order than hens, the latter were used exclusively.

A description based on the pecks administered or received does not give a very intimate picture of the actual social relations in a flock. As a result of observing birds, all of which are plainly marked to permit individual recognition, one finds definite personality traits which are not revealed by the more formal statistical summaries of the results of pair-contacts. A given individual may be hesitant, apparently fearful, or aggressive in different degrees; and its behavior may or may not vary in relation to the different social ranks of its associates. Dominant hens are characterized by the lack of any attempt to avoid other members of the flock; they may ignore the others, or they may chase and peck them. Only exceptionally are they pecked in return.

Subordinates low in the social order tend to avoid birds of higher rank. They retreat when pecked or threatened by high-ranking birds, and may give distinctive sounds, as though in pain, when pecked severely. The lowest-ranking birds in three of the first four pens observed in this study showed objective signs easily interpreted as fear of their superiors. Such behavior depends, often, but not always, on the behavior of the superior birds and grades from a mere quickening of pace to a strong avoiding reaction, which may include dodging backward, turning, and speedy retreat with some vocalization, as though in fear.

The severity of the peck, when given, varies widely. Blood may be drawn from the comb; feathers may be plucked; or, on the other hand, the peck may be light. The attack is often aimed at the comb or the top of the head; but, as the subordinate usually dodges the blow, it frequently lands on the neck or even on the back or shoulders or not at all. Under these conditions the superior hen often renews the attempt to deliver an effective blow. The fowls are usually close together when pecking, but threats may be given from a distance of even 2 feet or more. Pecks are rarely attempted when the subordinate is too far away to be hit.

Pullets and hens do not always react to each other when they come close together. They may be feeding busily about the food hopper and may pay no attention to the presence of another individual. Similarly, when sunning or otherwise resting during the day and after they settle on the roost at night, pecking is usually suspended. Frequently contacts can be stimulated by placing some mixed grains or similar food among the litter on the floor of the pen.

Some birds, usually high in the pecking-order without being at the top, show little negative reaction to their superiors and, when pecked, generally give only a slight avoiding reaction. Some individuals react strongly to being pecked. They may retreat to the other end of the pen, the roost, or the top of the nest boxes, or may hide in some corner, stand still with the head held low, or even crouch low with wings half lifted and the tail depressed, i.e., squatting, as hens do before a rooster. It would be easy to read human emotions into these reactions, a temptation which not all writers on the subject have resisted successfully.

Revolts and reverses are rare among hens. When an inferior pecks a superior, the latter, if it reacts at all, appears "surprised" rather than "frightened." It does not retreat, except to gather itself for attack; it draws itself up, leaps forward, and administers vigorous return pecks. The subordinate hen, which initiated the revolt, retreats as a rule but may also stretch up as high as possible, in characteristic fighting pose. Then each hen may leap forward, beak toward beak with neck elongated and arched and neck feathers raised, and so attempt to peck down on the head or seize the comb of its opponent. Noble, Wurm, and Schmidt (1938) in their study of the social organization of the black-crowned night heron report what may be a foreshortened variant of this type of behavior.

The frequency of pair-contacts varies greatly with different individuals and also with the time of day for the same birds. Lorenz (1935, p. 361), speaking presumably of jackdaws, says that there is a certain tension between birds which stand close to each other, particularly between those which stand near the top animal, and "on the other hand birds high in the peck order are good-natured to the low ranking birds." We have some evidence that this is true in our flocks. With some exceptions, we have found that hens high in the social order tend to peck their more immediate inferiors more often than they do those lower in social rank. A good part of the explanation, but not all, is that the lower birds, especially those near the bottom of the peck-order, tend to avoid their dominant pen mates when these come too near. Then, too, birds that rank near together are more often in close competition with each other.

Some of the individual differences mentioned in the preceding paragraphs are illustrated by observations made in preparation for the injections to be reported below. In one pen, BB, the α-bird pecked its subordinates an average of 41 times each over a period of 42 days; while in another pen during the same time, BR, also an α-bird, pecked its inferiors an average of 126 times. BR had fewer subordinates to peck; and one of them, GG, always kept away from the mash hoppers until the other birds had left. Another evidence of the difference between these two top birds was that BR frequently drove all other birds away from the mash hopper in the mornings; BB was not seen to do this.

Other complexities, which are poorly revealed in the simple tabulation of pecks, include cases of apparent antipathy between individuals. In one of the preliminary studies, BR pecked GG 131 times and its only other subordinate, which, furthermore, was its immediate inferior, only 64 times, although it had more opportunity to peck the latter, since GG, the bottom bird of its flock, commonly kept at some distance from the other birds. RW was seen to peck RY 122 times, while during the same observation periods it pecked RR only 57 times; yet RR stood just below RY in social rank. This could be interpreted as an example of the suggestion made by Lorenz which has just been cited; if so, the α-birds are able to draw a fine line in their behavior toward closely ranking subordinates. Discrepancy of treatment is also indicated by the observation that some superiors allowed some inferiors, but not all, to peck food from their bills.

Sickness limits the social activity of a bird and may result in a loss in social position. Unfortunately, we have had a considerable amount of experience with this factor. Of the original 24 top-crossed pullets which were received October 19, 1937, only 3 are now living. The majority of those that died have been autopsied by the veterinary department of Iowa State College,[3] and, of these, almost all died of fowl leucosis, with which the animals were infected when first received.

Evidence has been accumulating of a partial hormonal control of the aggressive reactions on which the social position of birds is based. This led Allee in 1936 to suggest the use of sex hormones in analyzing the underlying physiological basis of the social order in flocks of birds. Briefly, some of the evidence is: With domestic fowls, and with many other dimorphic species in which the male is larger and more showy, the male normally stands above the females in the social order. Domm and Van Dyke (1932) had found that injection of hebin, an extract of the anterior pituitary which contains LH and FSH as well as thyrotropic hormone, into young male leghorns causes great precosity in the development of head furnishings and also induces crowing (9 days), fighting, and treading by the age of 13 days. Capons certainly do not fight as vigorously as do cocks; in fact, Domm's experience (1927) led him to believe that a "gonadless" male neither crows, treads, nor fights. Benoit (1929) cites a decided exception to this general rule, and one of us has seen brief capon fights in Professor Koch's capon colony. Shoemaker, as early as 1936, had evidence that the social position of an individual canary tended to change during the progress of its breeding cycle.

These and other indications suggested that female aggressiveness might be enhanced by the injection of male hormone. The work of several investigators had shown (see Witschi and Miller, 1938, for a summary) that hens produce male hormone; and Gustavson (1932) prepared an extract from hen feces which stimulated comb growth in capons, a reaction now generally agreed to be produced only by androgenic stimulation. The injection of more male hormone would not mean the introduction of a totally strange material into the female system.

EXPERIMENTAL MATERIAL

Four lots of pure-bred white leghorn pullets or hens have been used in these studies. Their constitution and previous history follows:

Flocks A 1, A 2, A 3, and A 4.—These were top cross white leghorns procured from Dr. N. F. Waters, of Iowa State College. Each of these A flocks was composed of full sisters, the offspring of a random-bred hen mated with an inbred sire. The inbreeding coefficients of the sires used were 52.5, 67.0, 52.1, and 76.8 per cent, respectively. This means that the amount of inbreeding ranged from approximately four to something less than eight generations of brother-sister matings. Their breeding history is given by Waters and Lambert (1936). These pullets were approximately 6 months old when received in October, 1937. They were housed, 6 to a pen, in newly rebuilt and carefully fumigated pens in the Whitman Laboratory chicken-house. The pens were 14.5 feet long by 4.5–5.0 feet wide and about 8.0 feet high. Each pen is provided with raised roosts over a solid board platform to catch the droppings. The cement floor of the pens is kept covered with litter, which is changed weekly. Small doorways open onto sun porches on the south side of the buildings. These porches have wire bottoms as well as sides and top, so that the animals are held well above the ground. Light comes to the

[3] We are indebted to Professor N. F. Waters and to Dr. E. F. Waller for making and reporting these autopsies.

pens from a window at the south end of each; and, in addition, a 60-watt bulb hangs over the center of each pen. These are operated on a time switch and provide extra illumination during morning and evening hours during the winter. Winter temperature was controlled by a thermostat and ranged between 10° and 15° C. Our four pens were completely separated from other parts of the chicken-house and were on separate thermostatic and light controls. Our compartment was entered from the outside by its own door, which opened into a narrow corridor that gave direct access to each pen.

Each bird carried numbered wing and leg bands. For our purposes these were supplemented by one or more colored leg bands the colors of which gave the designations of the different individuals. GY, for example, wore a green and a yellow leg band. The subscript which is added at times indicates the flock number.

Almost immediately after receipt, flocks of the A series were placed under observation to determine the normal peck-order. These preliminary observations lasted from October 24 to December 24, 1937. Between that date and April 7, 1938, they were used in a set of experiments to determine the effects of injection of epinephrine.

As a result primarily of fowl leucosis, only 16 of the original 24 birds were alive on April 8. At or before that date the remnants were combined into two flocks, which were appropriately designated "A 1, 2" and "A 3, 4." The former contained 9 and the latter 7 individuals; further deaths reduced these numbers to 6 and 5, respectively, by the end of the first experiments with testosterone propionate, hereafter referred to as TP.

Flocks A 1, 2 and A 3, 4 were given some weeks under daily observations until the peck-order became stable. Injections of TP lasted from June 6 through July 29 (54 days in all), after which postinjection observations were continued until August 16. The 9 healthy birds still remaining were combined into flock A 5 and watched for 2 weeks; and selected birds were injected for 51 days, beginning August 30. After 3 weeks of postinjection observation only 5 were left. These were combined with 2 old hens recently received from an Indiana flock that had been line bred for 18 years without the introduction of new stock. This combination will be called flock "A 6."

Flocks B 1 and B 2.—These were originally composed of 24 inbred white leghorns from Iowa State College equally divided between their families 4 and 6 (Waters and Lambert, 1936). The individuals hatched out between January 3 and 31, 1938, and were received by us in two lots on March 19 and April 9, respectively, when they were 2–3 months old. Family 4 had at that time an inbreeding coefficient of between 68.0 and 73.6, and Family 6 had one of 59.3. Each of our B flocks contained 6 pullets from each family. In 1936 the birds in these two families had an *inter se* relationship of 62; the full sibs of Family 4 had a relationship of 88; and of Family 6, one of 74. The B flocks were housed 12 together in pens vacated by combining the A flocks. They were placed under observation immediately on receipt in order to study the establishment of the peck-order. Half of flock B 1 was removed for ovariotomy on June 22 and 23 and was permanently returned to the flock on July 19.

Injections of TP were made into selected individuals of flock B 2 from October 19 to December 21, 1938. On January 13, 1939, only 6 of this flock were still alive. Those autopsied showed no fowl leucosis. There were diagnoses of ulcer of the gizzard or proventriculus or erosion of the gizzard (4 cases) and an infected air sac (1 case). In flock B 1, 4 operated and 4 unoperated controls were alive on the same date. Those autopsied showed: acute hepatitis with fatal hemorrhage from rupture of the liver; abscess on breast muscle and a discolored liver, apparently secondary to abscess, since this bird was never injected.

Flocks C 1, C 2, and C 3.—These were also composed of pure but random-bred white leghorns. They were battery reared in our laboratory from newly hatched chicks which we received September 19, 1938, from the Illinois hatchery, of Metropolis, Illinois. Later they were housed 6 to a battery 40 inches long by 24 inches wide by 18 inches high (C 1 and C 2) and six more in one 28 inches long by 22 inches wide by 24 inches high (C 3). The former were kept at 20°–21° C.; the latter at 6° C. They had not been used in previous experiments before the present work began. The chickens received TP injections from December 10, 1938, to January 30, 1939.

Flock D 1.—This flock was originally composed of 12 aged hens of which 2 were taken to make flock A 6. They were received October 22, 1938. Something of the ruggedness of the stocks and the adequacy of our treatment is seen by the fact that, beyond the death of 1 bird in each of the C and D flocks almost immediately on receipt, all the rest were alive and apparently well in late February, 1939.

Care and feeding.—The A, B, and D flocks were fed and cared for by Mr. Collias except for brief periods, when Miss Lutherman acted as substitute. These two also made all the primary observations. While no other person has handled or cared for these flocks, Dr. L. V. Domm and his experienced caretaker, Mr. Emil Stange, have been extremely courteous in keeping watchful eyes on the welfare of our chickens and in giving advice concerning their care. The diet and treatment have been carefully based on the experience of Dr. Domm with his much more extensive colony.

Briefly, they were fed moist mash containing cod-liver oil in the morning and coarser, mixed grain in the afternoon. A hopper of dry mash was always present in each pen. Green food (cabbage or lettuce) was constantly present off the floor at the side of the pen. Water was before the birds at all times, and the containers were cleaned daily. Oyster shell, crushed limestone, and charcoal were always available to the birds.

The C flocks have been constantly under the care of Miss Lutherman. They have been fed Conkey's starting mash, growing mash, and mixed grains and have also had water constantly before them. In place of cod-liver oil they have been irradiated from 1 to 2 hours daily with light containing about the amount of ultra-violet found in noon sunlight.

Methods of observation.—In the A and B flocks behavior records throughout have been made by an observer sitting quietly, notebook in hand, in a neighboring pen, partly screened from the sight of the birds by the solid wooden partition that makes up the lower part of the walls of each pen. Only large-meshed chicken wire was present to interfere with the view of the near-by birds. Although easily disturbed when first placed under observation, the successive flocks were readily conditioned to the presence of the observer, as was shown by the resumption of normal activities even in the pen in which the observer was seated.

All pair-contacts were initiated by the birds themselves without stimulation other than by the fresh distribution of food. During the period of these experiments the birds were under observation 3–5 hours daily except that on Saturdays, when the pens were cleaned, and on Sunday observations were curtailed or omitted. Observations were made during one or two morning periods between 8:00 and noon and an afternoon period between 2:00 and 6:00. The B flocks were observed by the same method; also, they, together with flocks A 5 and A 6, were placed together in a neutral pen, pair by pair, for observation of initial contacts. The details of this procedure will be given later. Flocks C 1 and C 2 were watched from a distance of 4–6 feet; under the conditions that prevailed one could see the bands on the birds in the two middle batteries. So far, up to 6 months

of age, it has been possible to observe all birds of both pens at once and to record all pecks. Periods of observation of these C flocks have varied from 15 minutes to an hour, with 15–20 minutes per day being the more usual time spent. The first peck was observed when the birds were about 2.5 months old. During the early weeks, before pecking started, there was little to observe that is of interest from the present point of view. Filling the feed troughs just before the observation period is of little importance with these battery-housed birds, since they do not "fight" over the opportunity to feed. It was difficult to make accurate observations in the cold room because of the small space there.

OBSERVATIONS

Flock A 1, 2.—As constituted on April 8, this flock contained 9 hens. These were observed for 7 weeks before TP injections were begun; during the last 6 weeks of this preliminary period the social order had remained stable and is summarized in Table 1. This

TABLE 1

THE SOCIAL ORDER IN FLOCK A 1, 2 BEFORE TREATMENT

Individual	Number Pecked	Individuals Pecked							
BB	8	RG	RR	GG	BR	YY	BY	GY	RW
RW	7	RG	RR	GG	BR	YY	BY	GY	.....
GY	6	RG	RR	GG	BR	YY	BY	.....	.....
BY	5	RG	RR	GG	BR	YY	.....	.....	.....
YY	4	RG	RR	GG	BR	.....	.....	.....	.....
BR	3	RG	RR	GG	.....	.....	.....	.....	.....
GG	2	RG	RR	.....	.....	.....	.....	.....	.....
RR	1	RG	.....	.....	.....	.....	.....	.....	.....
RG	0	.....	.....	.....	.....	.....	.....	.....	.....

flock had organized itself in a pure-line system or simple hierarchy. The birds indicated by bold-faced type were selected for injection with the male hormone.[4] All injections were given daily and were made intramuscularly into the pectoral muscle. The locations of the punctures were varied systematically. The hormone was administered as received in sesame oil, and all other birds in the same flock were similarly injected daily with 0.1 or 0.2 cc. of sesame oil. In the present experiment **RG** received 1 mg. and **BR** 0.5 mg. of TP daily. As a check on the effectiveness of the hormone, Dr. L. V. Domm kindly consented to make independent injections of our TP into one of his long-time capons. According to expectations, there was prompt and vigorous comb growth.

Four of the hens died during the period of the experiment: BB on June 7, of fowl leucosis; GG on June 19, of impaction of crop and gizzard, RR on July 3, of fowl leucosis; and GY on August 1 (no diagnosis). BY died approximately 3 weeks after injections stopped. The reversals over these birds that died and even over BY may have been made more certain by reason of the ill-health of the losing bird. This cannot always have been

[4] The testosterone propionate (TP) was donated by the Ciba Pharmaceutical Products Company, Lafayette Park, Summit, N.J., and by the Schering Corporation, Bloomfield, N.J. Without their kind co-operation this work could not have been done. We detected no difference between the hormone furnished by the two companies.

an important factor, however, for BB maintained her position at the top of the pre-injection order up to her death. The ranking of the reduced flock on August 1, 2 days after injections stopped, is given in Table 2.

The basic observational data on which earlier tables are based are given in Table 3 in the form of summaries of the observed pecks for stated intervals which are arbitrarily chosen to give the maximum of information without making the table too long and complicated. Another observer was added during the fourth week after injections were started. The unsuccessful revolts that were observed are shown by asterisks, and the approximate date of each reversal can also be estimated. None of the reversals was actually observed. All the return pecks that are recorded for the first period actually were given on the first day that flocks A 1 and A 2 were united. The dramatic thing shown by the tables is the rise of **RG** from the bottom to the top position in the flock. This hen stood fifth among the 5 survivors of flock A 1. There is evidence that it did not submit passively to the lowly position which it occupied in flock A 1, 2. After the first day, however, no

TABLE 2

THE SOCIAL ORDER IN FLOCK A 1, 2 AFTER 54 DAYS OF INJECTIONS

Individual	Number Pecked	Individuals Pecked				Former Rank*	Formerly Pecked*
RG	3	BY	.....	YY	RW	5	0
RW	3	BY	**BR**	YY	.....	1	4
YY	2	BY	**BR**	.....	.....	3	2
BR	2	BY	.....	.....	RG	4	1
BY	0	.....	.....	.....	.....	2	3

*Among those still living.

overt indication of revolt was seen in the long period before injections started, and it was not until these had been going on for 4 weeks that **RG** began its social climb. **BR** successfully revolted against GY almost 5 weeks before the death of the latter, against BY 6 weeks before she died, maintained her dominance over **RG**, and staged one observed unsuccessful revolt against RW.

Flock A 3, 4.—On March 19 the remnants of former flocks A 3 and A 4 were combined into a flock of 7 individuals. No reversals in position occurred in the 11 weeks preceding injection of male hormone, which, as with the preceding flock, began on June 6. The dominance-subordination pattern of the flock at that time is shown in Table 4. The social order in this flock illustrates another basic type from that shown in Table 1. Here the top bird, GY, dominated all the others except RG, which, although it stood third from the bottom, had managed to get and hold superiority over GY. As shown again by bold-faced type, the lowest-ranking hens were injected daily with TP, **GG** with 0.5 mg. and **BG** with 0.25 mg. As always, the others were similarly injected with sesame oil. Two birds died during the injection period;[5] the ranking of the remaining members of the flock on August 1 is shown in Table 5.

Again no successful revolts were actually seen, but the daily observations permitted these to be dated with confidence. The unsuccessful revolts that were observed and the

[5] RG on June 16, of fowl leucosis; GY on July 19, no diagnosis but with "no enlargement of organs as expected in leucosis."

TABLE 3

SUMMARY OF OBSERVED PECKS IN FLOCK A 1, 2 BEGINNING APRIL 8

Hens injected with hormones and pecks in the reversed direction are shown in bold-faced type. Double vertical lines inclose the period of injection. Asterisks mark unsuccessful revolts.

DOMINANT	SUBORDINATE	DAYS SUMMARIZED								PECKS	REVERSED PECKS
		10	21	28	14	14	14	12	18		
RW	GY	1	4	8	25	15	11	3	Dead	67	0
	BY	3 6	*10	17	4	3	5	5	13	63	**3**
	YY	2	3	3	3	1	5	4	9	30	0
	BR	1	12	14	*13	13	11	1	19	84	**2**
	GG	2	8	8	6	Dead	.....	.....	.....	24	0
	RR	1	11	5	9	5	Dead	.....	.....	31	0
	RG	1 1	12	4	0	4	1	**2**	**62**	22	**65**
GY	BY	1	3	4	15	8	0	6	.....	37	0
	YY	6	2	6	14	3	0	4	GY	35	0
	BR	5	2	8	4	1 **46**	**26**	**8**	Dead	20	**80**
	GG	5	1	1	3	Dead	.....	.....	.....	10	0
	RR	1	4	1	24	1	Dead	.....	.....	31	0
	RG	**6** 2	0	2	*7	6	1	**3**	.....	19	**10**
BY	YY	14	23	15	38	15	5 **9**	**15**	**8**	110	**32**
	BR	9	14	8	8	8	**12**	**1**	**9**	47	**22**
	GG	7	11	2	0	Dead	.....	.....	.....	20	0
	RR	5	24	9	24	7	Dead	.....	.....	69	0
	RG	1 8	7	10	6	5	1 **26**	**11**	**20**	37	**8**
YY	**BR**	5	36	5	19	10	20	12	10	117	0
	GG	3	16	6	9	Dead	.....	.....	.....	34	0
	RR	6	8	8	13	2	Dead	.....	.....	37	0
	RG	**3** 2	8	6	9	5	3 **19**	**25**	**58**	33	**105**
BR	GG	3	11	12	2	Dead	.....	.....	.....	28	0
	RR	4	34	26	37	8	Dead	.....	.....	109	0
	RG	2	13	18	6	27	3	1	6	76	0
GG	RR	3	31	4	5	GG	Dead	.....	.....	43	0
	RG	**2** 2	13	12	0	.....	.....	.....	.....	27	**2**
RR	**RG**	2	1	3	8	1	RR	Dead	.....	16	0
RG	0	0	0	0	0	.....	0	0	0	0	0
Totals	.....	127	323	227	311	194	158	101	214	1,276	**379**

TABLE 4

THE PECK-ORDER IN FLOCK A 3, 4 BEFORE TREATMENT

Individual	Number Pecked	Individuals Pecked						
GY	5	BG	GG	.....	BY	BR	RR	.....
RR	5	BG	GG	RG	BY	BR	.....	.....
BR	4	BG	GG	RG	BY	.....	.....	.....
BY	3	BG	GG	RG	.....	.....	.....	.....
RG	2	.....	GG	.....	.....	.....	.....	GY
GG	1	BG	.....	.....	.....	.....	.....	.....
BG	1	.....	.....	RG	.....	.....	.....	.....

other basic data are summarized in Table 6, as in Table 3. In this flock again both injected birds moved up in the social scale: **GG** went from next to the bottom to the top,

TABLE 5

THE PECK-ORDER IN FLOCK A 3, 4 AFTER 54 DAYS OF INJECTIONS

Individual	Number Pecked	Individuals Pecked				Former Rank*	Formerly Pecked*
GG	4	BY	BR	**BG**	RR	4	1
RR	3	BY	BR	**BG**	.....	1	4
BG	2	BY	BR	.....	.....	5	0
BR	1	BY	.....	.....	.....	2	3
BY	0	.....	.....	.....	.....	3	2

* Among those still living.

TABLE 6

SUMMARY OF OBSERVED PECKS IN FLOCK A 3, 4 BEGINNING MARCH 19

Hens injected with hormone and pecks in the reversed direction are shown in bold-faced type. Asterisks mark unsuccessful revolts. Double vertical lines inclose the period of injection. BG was added on April 30.

Dominant	Subordinate	Days Summarized									Pecks	Reversed Pecks
		8	28	14	28	14	14	14	12	18		
GY	RR	19	17	15	47	29	46	16	GY	.....	189	0
	BR	18	17	20	19	11	*20	7	Dead	.....	112	0
	BY	12	20	4	17	25	16	11	.....	.....	105	0
	GG	15	13	7	21	10	*16	*10	.....	.....	92	0
	BG	.....	.....	16	41	19	18	35	.....	.....	129	0
RR	BR	***30	33	18	27	16	13	2	11	22	172	0
	BY	40	24	19	33	41	38	18	19	2	234	0
	RG	31	32	9	38	19	Dead	.....	.....	.....	129	0
	GG	18	17	15	24	21	15	*9	7	113	126	**113**
	BG	.....	.....	27	65	22	55	76	82	55	382	0
BR	BY	6	10	5	6	7	*1	12	15	8	70	0
	BG	24	51	25	21	12	Dead	.....	.....	.....	134	0
	GG	5	17	8	17	18	*1 **22**	**21**	**37**	**57**	67	**137**
	BG	.....	.....	36	57	19	3 **11**	**18**	1 **33**	1 **52**	117	**114**
BY	RG	20	15	3	6	14	Dead	.....	.....	.....	58	0
	GG	0	0	3	1	*8	**12**	**2**	**8**	**23**	12	**43**
	BG	.....	.....	2	5	4	**22**	**44**	**54**	**5**	11	**125**
RG	GY	24	21	5	21	22	RG	.....	.....	.....	93	0
	GG	14	10	7 1	*19	18	Dead	.....	.....	.....	68	1
GG	**BG**	.....	.....	39 **2**	46	28	23	14	25	40	215	**2**
BG	RG	.....	.....	2	10	20	Dead	.....	.....	.....	32	0
Totals	.....	276	297	288	542	383	332	295	293	376	2,547	**535**

and **BG** staged successful revolts against BY and BR. It is worth noting that GY maintained its dominant position until its death and was able to put down attempted revolts which were made on three different occasions by **GG**; also, that BR was broody during

the injection period, and its comb, and presumably its supply of male hormone, became much reduced.

Flock A 5.—This flock was composed of the 9 remaining birds from the original A flocks. They were placed together on August 17 and were watched then for the first 5 hours, which is the period most crucial for the establishment of the social order. As it happens, in this flock there were no reversals after this initial period for the first 4 weeks that

TABLE 7

THE SOCIAL ORDER IN FLOCK A 5 BEFORE TREATMENT

Individual	Number Pecked	Individuals Pecked								
RG	7	BY	RY	BG	RR	GG	.....	YY	RW	.....
RW	7	BY	RY	BG	RR	GG	BR	YY	.....	.....
YY	6	BY	RY	BG	RR	GG	BR	.....	.....	.....
BR	6	BY	RY	BG	RR	GG	.....	.....	.....	RG
GG	4	BY	RY	BG	RR	.....	.....	.....	.....	.....
RR	3	BY	RY	BG	.....	.....	.....	.....	.....	.....
BG	2	BY	RY	.....	.....	.....	.....	.....	.....	.....
RY	1	BY	.....	.....	.....	.....	.....	.....	.....	.....
BY	0	.....	.....	.....	.....	.....	.....	.....	.....	.....

TABLE 8

THE PECK-ORDER IN FLOCK A 5 AFTER 51 DAYS OF TREATMENT OF THE HENS SHOWN IN BOLD-FACED TYPE

Individual	Number Pecked	Individuals Pecked									Former Rank	Formerly Pecked
BY	7	**BG**	RR	GG	.....	BR	YY	RW	RG	.....	9	0
RG	6	**BG**	RR	GG	**RY**	.....	YY	RW	.....	.....	1	7
RW	6	**BG**	RR	GG	**RY**	BR	YY	.....	.....	.....	1	7
YY	4	**BG**	RR	GG	.....	BR	.....	.....	.....	.....	3	6
BR	4	.....	RR	GG	**RY**	.....	.....	.....	RG	.....	3	6
RY	4	**BG**	RR	.....	.....	.....	YY	.....	.....	BY	8	1
GG	3	**BG**	RR	.....	**RY**	.....	.....	.....	.....	.....	5	4
RR	1	**BG**	.....	.....	.....	.....	.....	.....	.....	.....	6	3
BG	1	.....	.....	.....	.....	BR	.....	.....	.....	.....	7	2

the hens were together—and then only by injected individuals. All the birds that received testosterone gave 1 or more reversals. There were no deaths during the progress of the experiment. The peck-order on August 30, when injections began, is shown in Table 7.

BG was given 0.75 mg., **RY** 1.00 mg., and **BY** 1.25 mg. of TP daily. **BG** was removed on October 4 (ninth period) because of illness, and her injections were discontinued. Otherwise the hormone was administered for 51 days. The flock organization at that time is shown in Table 8. Again the dramatic feature of the experiment is the climbing of **BY** from the bottom to the top of the flock, where she dominated all except **RY**, which

had also received substantial injections of male hormone. **RY** went up from eighth place to a tie for fourth, with reversals over RR and YY and **BG**. Despite illness, **BG** lost only to her pen mates that were also receiving the hormone, and gained a reversal over BR.

Two successful revolts were observed. On September 13 **RY** attacked her superior, YY; they fought briefly, and YY was beaten and retreated. Thereafter **RY** frequently asserted her superiority over YY. Fifteen days later **RY** engaged in a more elaborate encounter with her superior, RR. She fought with RR just after the latter was returned to the pen following injection with sesame oil. RR turned away and retreated, an action which marks a reversal if the fight is decisive. A little later **RY** leaped on RR, and they fought vigorously for a moment, then RR ran. **RY** kept picking up and dropping a small feather and calling. Later **RY** again jumped on RR in fighting pose and pecked her severely; RR retreated and ran. Still later **RY** gave RR another vigorous peck, and this time RR flew to a roost. After a time RR came down to the floor only to receive another peck from **RY**. All this happened between 4:00 and 4:30 P.M. Although these two birds had not been seen to fight that morning, RR had threatened **RY** during the earlier observation; hence, this probably was the complete story of the reversal, while the action between **RY** and YY may have been merely the final scene in a longer struggle, the early phases of which were not observed.

An unexplained pair-contact reaction was seen on October 10 at about 5:00 P.M. Earlier in the period of watching, GG had pecked **RY**, as was usual; then near the end of the observation period, **RY** and GG fought actively for 5 or 10 seconds, during which time **RY** seized GG by the comb and threw her down. GG got away and retreated beneath the roosts, where she remained for some time. **RY** did not follow but flew to the top of the nest boxes and stayed until the observer left. Apparently this had been an entirely successful revolt, but the next day GG was again pecking **RY** as though nothing had happened. The fact that the revolt occurred late in the day and that **RY** did not immediately follow up her advantage may have had something to do with the matter. Of course, there may have been another unwitnessed and crucial encounter which **RY** lost, especially since later observations of another reversal, not in connection with this experiment, showed that a series of revolts occurred before the dominant bird relinquished the peck-right to a revolting subordinate. During the series of combats still another bird, dominant to both, kept joining in and fighting against the initial rebel, almost as if aiding the defending dominant bird, which, nevertheless, in this case eventually lost its position. This illustrates one of the unavoidable difficulties of our work; in order to know all that happens, the hens should be under constant surveillance and yet, as has been indicated earlier, there would be hours and hours of such watching that would ordinarily be wasted.

The basic observations with flock A 5 are summarized in Table 9, which is arranged as were Tables 3 and 6.

Since the other experiments to be reported involved other stocks of birds, at least in part, and used other techniques, a summary of the effects produced in these tests which were made on the A flocks is given in Table 10.

During the same period of time, of the 12 individuals similarly injected with sesame oil alone, only 1 reversal was given. This was by YY over BY in flock 1, 2 and took place after BY had lost to its 2 hormone-injected flock mates; BY died just after the close of the experiment. Of the 22 reversals given by the hens receiving hormones, only 5 are less important, because they were made over a bird which may have been ill. On the

other hand, BG in flock A 5, the only one of these injected individuals to become ill while receiving hormone, gained and kept 1 reversal shortly before she was removed because of illness.

TABLE 9

SUMMARY OF OBSERVED PECKS IN FLOCK A 5 BEGINNING AUGUST 17

Hens injected with hormone and pecks in the reversed direction are shown in bold-faced type. Double vertical lines inclose the period of injection. Asterisks mark unsuccessful revolts.

Dominant	Subordinate	Days Summarized								Pecks	Reversed Pecks
		1	12	13	7	7	7	17	18		
RG	RW	22	20	5	2	1	0	6	1	57	0
	YY	17	25	26	4	11	11	5	8	107	0
	GG	1	8	12	15	9	2	16	3	66	0
	RR	.....	17	8	9	1	6	4	3	48	0
	BG	9	19	16	10	13	13	7	0	87	0
	RY	1	11	10	8	4	1	*11	6	52	0
	BY	.....	13	12	30	15	*6	1 **98**	**62**	77	160
RW	YY	7	2	13	2	10	7	20	8	69	0
	BR	9	6	8	14	27	6	36	5	111	0
	GG	7	6	5	12	1	1	20	5	57	0
	RR	12	*9	11	3	2	4	2	1	44	0
	BG	7	1	11	5	4	8	2	0	38	0
	RY	12	5	10	5	2	1	15	5 **3**	55	3
	BY	10	3	14	27	16	5	8 **42**	**34**	83	76
YY	BR	7	15	13	2	18	31	65	13	164	0
	GG	3	1	0	0	5	1	36	5	51	0
	RR	5	13	6	0	3	4	3	2	36	0
	BG	8	*8	5	5	13	10	9	6	58	0
	RY	4	11	3	3 **4**	**21**	**13**	**27**	**18**	21	83
	BY	4	2	3	2	**13**	**3**	**60**	**27**	11	103
BR	RG	2	3	12	5	4	4	22	1	53	0
	GG	4	15	13	2	9	18	82	13	156	0
	RR	44	9	*5	2	6	16	12	4	98	0
	BG	9	6	7	1 **1**	2	**8**	1 **1**	0	26	10
	RY	7	14	7	3	23	24	47	17	142	0
	BY	4	25	12	25	35	9 **1**	**102**	**52**	110	155
GG	RR	5	3	12	8	8	17	3	2	64	0
	BG	.....	1	15	6	21	48	14	0	105	0
	RY	.....	2	7	16	17	21	*46	15	124	0
	BY	.....	4	6	29	14	16	133	19	198	23
RR	**BG**	.....	2	3	3	4	19	5	0	36	0
	RY	.....	5	2	*7	7	12 **8**	**3**	**6**	33	17
	BY	.....	3	5	*6	9	**10**	**5**	**2**	23	17
BG	**RY**	2	5	3	2	**6**	**23**	1	0	12	30
	BY	.....	4	3	8	1	**4**	0	0	15	5
RY	**BY**	.....	3	7	7	10	14	71	62	174	0
BY	.....	0	0	0	0	0	0	0	0	.....	.....
Totals	.....	222	299	310	293	365	405	571	402	2,661	682

In general, as Table 10 shows, there were more reversals as the dosage became larger. **BG** again is an exception on this point; it was also the only individual receiving male hormone which allowed other hormone-injected birds to rise above it in social status. Both

BR in flock A 1, 2 and **RY** in flock A 5 retained the peck-right over their injected associates **RG** and **BY,** respectively, which otherwise went to the top of their social groups.

As the more detailed tables show (3, 6, and 9), there was a latent period before the injected birds began to stage revolts, and the beginning of reversals came still later. Reversals were usually made first over the fowls low in the peck-order, and only near the end of the prolonged injection periods, if at all, over the ranking birds in the respective flocks.

As was to be expected, TP produced other physiological effects besides those reflected directly by changes in social position. Certain of these will be discussed later, when all such effects will be considered together.

Flock B 2.—These birds were placed under continuous daily observation about the time that they were first beginning to peck each other. During the early months of observation, social position in this particular flock of highly inbred birds was more unstable

TABLE 10

THE EFFECT OF MALE HORMONE ON SOCIAL POSITION IN THE A FLOCKS

Flock	Bird	Daily Dosage (Milligrams)	Number of Reversals
A 3, 4	**BG**	0.25	2
	GG	0.50	3
A 1, 2	**BR**	0.50	2
	RG	1.00	4
A 5	**BG***	0.75	1
	RY	1.00	3
	BY	1.25	7
Totals	7	.....	22

* Became ill during experiment.

than that of any other flock of domestic fowls of which we have record. The last spontaneous reversal occurred during the twenty-seventh week of observation; the next to the last happened 4 weeks earlier. Thereafter the social order remained stable. Eleven of the 12 original birds were alive at the beginning of injections. Three control members of the flock and two that were receiving injections (**BB** and **GG**) died during the injection treatment. The peck-order of those that survived throughout and all those injected with TP is shown in Table 11 at the beginning of injections. As before, the individuals listed at the left in bold-faced type were given the male hormone daily; the others received 0.2 cc. of sesame oil at the same time. **GG** was given 1.25 mg. of TP from October 19 through December 4; **BB** received 1.0 mg. daily for the same period. **RW** was injected with 0.75 mg. each day until December 4, when its dosage was doubled for the following 17 days. **GG** and **BB** died about a week after their removal from the experiment on December 4.[6]

No reversals were given by any of the birds in flock B 2 during the period of injection. Four unsuccessful revolts were seen: 3 by birds that were receiving hormone, and 1 by a

[6] Autopsies at Iowa State College showed erosion of the gizzard and extreme emaciation of both; BB also had an egg free in her body cavity.

control. Nineteen reverse pecks were seen; 7 were delivered by 2 of the 3 pullets injected with TP, and 12 by 5 controls. In other effects, as will be seen later, injections in this flock produced physiological results similar to those in the A flocks, and, as will be told in the next section, the injected birds showed definite behavior effects as a result of receiving the hormone. Under these conditions we have no complete and certain explanation of the failure to induce social reversals such as had been given by each of the previously injected birds. Since crowing was induced early in the bird receiving the highest dosage and was discontinued in a short time, it is possible that these young birds became somewhat refractory as a result of continued treatment with heavy dosage. The primary difference between these birds and the others appears to be in age; and it is to be noted that previous investigators (Hamilton, 1938), working with young chicks, did not get a crowing response in the female by injection of male hormone. Since there were no reversals, there is no need to exhibit the basic data. Approximately 4 months later we were able to produce a typical set of reversals in this flock by another series of injections (see p. 434).

TABLE 11

THE SOCIAL ORDER IN FLOCK B 2 BEFORE TREATMENT

Individual	Number Pecked	Individuals Pecked						
BG	7	GG	RW	BB	YY	RR	BR	GY
GY	5	GG	RW	BB	YY	.....	BR	.....
RR	5	GG	RW	BB	YY	.....	.....	GY
BR	4	GG	.....	BB	YY	RR	.....	.....
YY	2	.....	RW	BB	.....	.....	.....	.....
BB	2	GG	RW	.....	.....	.....	.....	.....
RW	2	GG	.....	.....	.....	.....	BR	.....
GG	1	.....	.....	.....	YY	.....	.....	.....

INITIAL CONTACTS

The observations reported so far were made by watching the reactions of the birds when engaged in their normal activities in the flocks of which they were constantly members and in which each had a well-established social niche. A revolt or reversal was therefore made in the face of all the social inertia inherent in such a flock organization. As has been shown in the introductory pages, social position among these birds is established by the outcome of initial contacts and, once established, is rarely reversed.

In connection with other studies we devised a system of experimentally induced initial contacts. The typical procedure for Series I was to scatter some scratch food on the floor of the observation pen; then one person caught one of the birds, while another caught the other. Each individual bird came from a separate flock; in the cases we are now considering, only the injected birds had ever been in contact with members of the other flock, and with them 5 weeks or more had elapsed before the contacts were repeated. Each fowl was weighed, the state of its plumage and general health was noted, position in the social order in the home flock was recorded, and standard comb measurements were taken. Length of comb was measured along the greatest length of the blade; height was measured for the point which stood highest and most perpendicularly above the head. Successive measurements on the same individual were made on the same point. Finally

a prediction was entered concerning the outcome of the pair contact which was about to be staged. One observer took a bird under each arm, while the other prepared to make and record observations. The chickens were introduced into a strange pen, which was purposely reduced to half the standard size to insure closer association.

In Series I, five sets of such initial contacts were staged. Set I was between fowls in flock B 2 which had been selected for injection and all those in flock B 1. This latter flock was a wholly similar flock of inbred birds of the same age; the only difference was that 5 of the 10 birds then in flock B 1 were poulards. Set II was between all members of these two flocks and was begun 32 days after injections were started. By that time the comb approached its maximum height. Set III consisted of pair-contacts between the survivors of A 5 and the 2 old Indiana hens which were to be combined with them to make flock A 6. They were given these controlled initial contacts on neutral territory; and, when permanently placed together 5 days after the first pair-contact of the series, they took the position in the social order which was indicated by the outcome of the staged initial encounter. Set IV was a series of staged encounters between members of flock A 6 and the B flocks. Set V includes all staged contacts in the C flocks.

Series II was run by one observer alone with as few modifications as feasible from the routine in Series I. The principal difference was that in the earlier series the same bird was never introduced with another on successive days, while in Series II most birds were so introduced every day. Their opponents, however, always varied from day to day. The contacts in Series II were staged from January 11 to February 27 in connection with studies on the effect of estradiol on the social order and were begun 21 days after the last injection of TP.

Although available in tabular form, the full details will not be given. Many of the data may be used later in another connection, and only those are presented here which bear directly on the role of male hormone in determining social position. Originally this series of observations was begun to test whether there is a relation between the size of the comb among closely inbred normal pullets and the result of the socially important first encounter between two strange birds. The observations were extended to include all other normal individuals together with operated and hormone-injected birds, to allow as complete a test as possible. The results are summarized in Table 12. Some of the general findings are: Poulards with small combs, though often weighing more and standing higher in the social order of their home flock, lost all but 2 encounters. These 2 instances were won by the poulard BB, which had the smaller comb, was heavier, had the higher social rank, and won by passive submission of the other bird.

Pullets injected with TP, though low in social position and light in weight, won all but 4 of their pair-contacts. Such birds had, on the average, larger combs and presumably, therefore, had more male hormone in their system than did the birds to which they lost. The number of actual fights in these staged contacts was small; of the 124 encounters which were staged first, only 24 were settled by fighting. In the remainder, 1 bird retreated without a struggle, i.e., there was passive submission.

In 16 of the 74 cases in which poulards were involved, the bird with the larger comb lost. Two of these involved the poulard BB mentioned above, when for some unknown reason the normal birds submitted passively. The 11 such contacts listed in the table were between the poulard GG and the birds of flock B 2. At the time of these experiments GG resembled a masculinized poulard or a pullet on full injection dosage with TP, since its comb was much larger than that of its pen mates. On closer inspection,

small cocklike hackles could be detected on the neck and flanks, and small spurs were present. Later observations showed continued masculinization.

The contacts between this GG_1 and YY_2 and between GG_1 and BR_2 were unusual in that the first reactions were inconclusive. The third time GG_1 and YY_2 were placed together, the latter won. With BR_2 it was not until the fourth staged encounter that we decided that GG_1 was the loser. The protocols of these encounters are given in Table 13. Both these normal birds acted more warily in the presence of GG than when placed with other birds which they dominated. To human eyes at least, GG was not as aggressive as was to be expected from its appearance. These observations raise another interesting

TABLE 12

THE OUTCOME OF INITIAL CONTACTS ANALYZED ON THE BASIS OF SIZE OF COMB AS AN INDICATOR OF THE AMOUNT OF MALE HORMONE PRESENT

Series	Contact Pairs*	Bird with Larger Comb		Mean Size of Comb*†	
		Won	Lost	Won	Lost
1	Normal:Normal	32	13	128	115
2	Normal:Normal	33	12	150	133
1	TP:Normal	12	4	143	131
2	TP:Normal	22	2	148	133
1	TP:Poulard	12	2	156	89
2	TP:Poulard	3	0	156	107
1	Normal:Poulard	37	11	108	87
2	Normal:Poulard	6	3	184	103
2	TP:TP	0	2	142	146
2	Other combinations	81	35	145	131
Totals or true means		238	84	140±0.9	120±0.9

* One pair of birds, not included here, had combs equal in size.

† Length plus height.

possibility. The relatively sudden growth of turgid, hypertrophied furnishings on the head of a relatively slight hen present some handicaps for successful fighting. The delivery of effective pecks is probably rendered more difficult, and the possibility of being pecked on the comb is certainly increased. Many of the problems suggested by the changed appearance of injected hens, or of such operates as GG, both psychological and physiological, could be effectively attacked by the use of dubbed birds.

Of the four contacts lost by hormone-injected birds and won by normal controls, 2 fights each were won by 2 normals from the same 2 injected pullets. These normal individuals (BW and BY) had smaller combs than their injected opponents; they were, however, able and enduring fighters. One of the injected birds (**BB**) was losing weight rapidly at the time, and the other (GG_2) seemed to be blind in one eye; both were ill and died after about 3 weeks. Neither had the endurance of BW or BY, although each won all its remaining staged contacts with normal birds.

These experimental contacts provide another indication of the increase of aggressiveness in these fowls following TP treatment. **BB, RW,** and **GG** of flock B 2 lost 9 of their 12 contacts with BW, RW, RR, and BY of flock B 1 before injection started. These were the only ones tried at that time. **BB** and **GG** lost all 4 contacts, and **RW** lost 1 of 4. After 32 or more days of injection, and despite the fact that **BB** and **GG** were not very well at the time, **RW** won all 4 and the others each won 2 of their 4 contacts with the same birds. An adverse score of 9 losses and 3 wins before treatment was turned into a favorable one of 8 wins and 4 losses. Further, the operate BB_1 had won from $\mathbf{BB_2}$ and $\mathbf{GG_2}$ before injections were begun but lost to these as well as to **RW** while they were being given male hormone.

TABLE 13

A SYNOPSIS OF EXPERIMENTAL PAIR-CONTACTS BETWEEN THE POULARD GG_1 AND THE NORMAL HEN, BR_2

	I	II	III
Date	10-7-38	10-23-38	10-28-38
Weight, pounds	3.6:3.6	3.3:3.3	3.2:3.3
Comb, millimeters	55+101:42+76	56+97:32+67	56+103:30+67
	Large, red:Normal	As before	As before
Social position	8 :5	As before	As before
Molting	No :Yes	As before	As before
Start, P.M.	4:13	3:53	4:23
Behavior	GG avoids BR	GG feeds	BR threatens GG
	BR half-threatens GG	GG apparently avoids BR	GG avoids BR
	4:33, out, no contest	BR half-threatens GG	BR threatens GG
	5:04, in again	BR feeds	4:27, out
	GG avoids BR	BR threatens GG	BR judged to have won
	BR feeds	GG feeds	
	GG avoids BR	4:13, out, no contest	
	BR half-threatens GG	NOTE: BR is pecked by RW, which has large comb, and acts as though afraid of GG's appearance	
	5:22, out, no contest		

In general, we can conclude from these observations on the B flocks that, while the injections did not cause the lowest 3 birds to climb in the social order of their home flock, it did decidedly increase their aggressiveness and success in encounters with their near relatives in a parallel flock. Also, these staged initial contacts between normal pullets showed that, while the amount of male hormone present, as measured by comb size, is not an infallible guide to the winner of such contacts, it is a good indicator—in fact, one of the best single indicators we have found—of the probable winner.

The C flocks.—The whole treatment of the C flocks differs radically from that which we have been describing. These were battery reared in our laboratory. One of our interests with them was to study the onset of pecking in two flocks of 6 birds in separate batteries at 20°–21° C., as compared to those in another battery which, since their thirteenth week of age, had been kept at 6° C. The temperatures were chosen in connection with another experiment. The birds were hatched on September 18. RG, of flock C_2 in the warmer room, was seen to peck a battery mate on December 3. A week later, during which time no pecking had been seen, injections of TP were begun into **RR** in the warm room and **GG** in the cool room. **RR** was seen to peck another bird a week later (Decem-

ber 17) and continued to do so steadily. Injections were continued until January 20, 1939, when **RR** became the α-bird of the now firmly established peck-order.

BB of the same flock was first seen to peck another bird on December 17; it was not found pecking again until 4 weeks later. Between December 23 and 29, 5 more of the 11 controls in the warm room were observed pecking other birds. The smaller space in the cold room made observation difficult. No pecks were recorded for these pullets until after injections were begun; and then **GG**, the injected bird, did the first pecking that was seen. **GG** started a few days after **RR** began in the warm room, and has in general behaved much the same as **RR,** at least as nearly as can be detected. We recognize that these observations on the C flocks are inconclusive. For one thing, the injections did not begin until the most precocious bird had started to peck its fellows. However, the behavior is at least what would be expected from the observations on the other flocks.

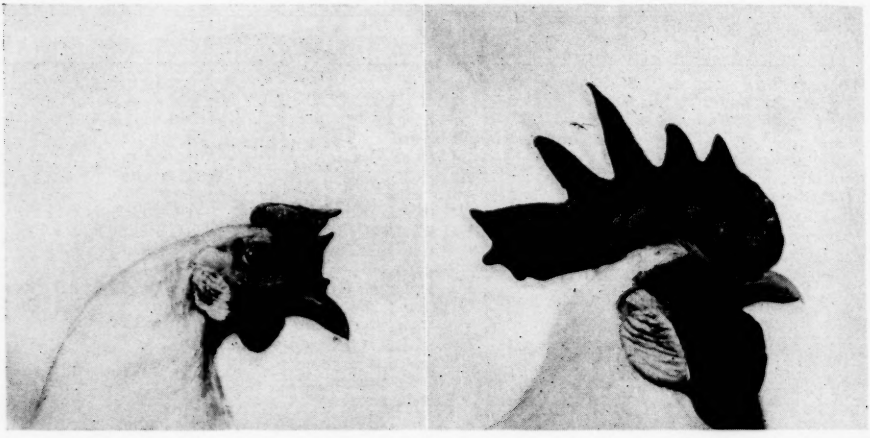

FIG. 1.—**BY** of flock A 5 before (*left*) and after 51 daily injections with testosterone propionate

OTHER EFFECTS

Comb growth.—As was to be expected, the comb and other head furnishings of the birds given TP started to grow immediately. A typical effect is given in Figure 1, in which the left photograph shows **BY** of flock A 5 on August 23 before injection with TP and the right photograph shows her on October 19, after having been injected with 1.25 mg. daily of TP for 51 days. In the meantime, as shown in Table 8, she had moved from the bottom to the top of the social order in her flock. Some general indication of the size of comb attained in comparison with uninjected flock mates is found in the fact that the birds in the A and B flocks showed a mean increase in length plus height of 38 per cent from the beginning to the peak size, while oil-injected controls increased only 2 per cent. A decrease set in at the close of the injections, which in flocks A 1, 2 and A 3, 4 amounted to 3.3 per cent on the sixth day following cessation of treatment. In the A flocks, the full comb size, as measured by length plus width, varied between 197 and 211 mm. The combs of the birds not given male hormone, which ranked highest in the social order of

each flock, ranged from 168 to 186 mm. The combs of flock B 2 were smaller; the three TP-treated pullets at full comb development ranged from 154 to 163 mm. at a time when their pen mates had combs which varied between 86 and 147 mm. The mean percentage increase among these injected birds of B 2 was 55 per cent, as contrasted with an average decrease of 7 per cent in average comb size among their controls. The comb of **RW,** the one treated bird to survive, decreased 9 per cent in the first 18 days following cessation of injection.

It is possible that the smaller total size of comb which was attained among these injected birds of this highly inbred flock may have been correlated with their failure to move up in the social scale. Against such a conclusion it must be considered that the percentage of difference in comb size between TP-injected birds and their controls was greater in this flock than it was in the A flocks, where each bird that received the hormone did move up in its social ranking and where one injected individual reached top position in each flock.

TABLE 14

EFFECT OF TESTOSTERONE ON WEIGHT

Flock	Days Injected	Number of Control Birds	Number Treated	Change in Weight (Ounces)		Percentage of Change	
				Control	Treated	Control	Treated
A 1, 2	54	3	2	− 1	+ 8	− 1.6	+12.3
A 3, 4	54	3	2	− 1	+ 3	− 1.6	+ 5.0
A 5	51	6	2	− 5	+ 0.5	− 7.7	+ 0.8
B 2	11	7	3	− 1.1	+ 1.5	− 2.0	+ 2.8
B 2	54	5	1	− 3.7	− 1	− 6.7	− 2.0
C	42	2	2	+18.5	+11.5	+59	+42

In the C flocks the combs of the 2 hormone-injected pullets increased 200 per cent during the period of treatment (December 10–January 20), and the comb of each showed a very slight but measurable decrease on the day following the cessation of injections.

Weight.—With young chicks Breneman (1939) found that those injected with TP showed no definite weight changes during the period of treatment but that the controls increased more in weight afterward than did the injected birds. On the other hand, Bulliard and Ravina (1938) found that, after 2 or 3 weeks of treatment with the same androgen, ducks about 2 months old showed a slight increase in weight with males and a proportionately greater increase with females.

Our experience on this point is summarized in Table 14, which shows that in the older birds (A and B flocks), in which the animals had wholly or approximately completed their growth when injections of TP were begun, the treated individuals gained weight or in one instance lost slightly, while the controls all lost weight. Considered as 4 paired comparisons, the differences in the first 4 cases have a statistical significance (P) of 0.032. With the young, rapidly growing birds of the C flocks the controls grew more rapidly; and, when these are considered together with the first four sets, the results are statistically insignificant ($P=0.37$). In so far as gain or loss of weight is taken as an indication of well-being, we would expect from these results that the hormone-treated individuals in the

A and B flocks were in better physiological condition than their controls and hence might come to have a higher social status.

Crowing.—Precocious crowing in young male chicks has been induced by the age of 60 hours by the administration of 20 mg. of TP in a single dose on the day after hatching (Hamilton and Dorfman, 1939). This marks a further speeding-up of the crowing reaction from earlier records of 10 or 11 days through the injection of TP (Hamilton, 1938; Breneman, 1938). Hamilton failed to secure crowing with female white leghorn chicks, although they were injected and observed for a period of 55 days. Our experience with the induction of crowing in pullets and hens is summarized in Table 15. Again, it must be remembered that our observations cover only a part of each day, and we have no means

TABLE 15

EFFECT OF TESTOSTERONE ON CROWING

Flock	Individual	Days Injected before Crowing	Days Crowing Continued after Injection	Daily Dosage (Milligrams)	Remarks
A 1, 2.....	RG	38	3	1.0	Crowed over 60 times
	BR	.....	.....	0.5	Not observed to crow
A 3, 4.....	GG	45	.....	0.5	Crowed 8 times on forty-fifth day; not heard later
	BG	.....	.....	0.25	Not observed to crow
A 5.....	BY	30	3	1.25	Crowed at least 74 times
	RY	37	.....	1.00	Crowed at least 20 times; not heard after injections ceased
	BG	.....	.....	0.75	Not observed to crow (became ill)
B 2.....	GG	16	.....	1.25	Crowed 11 times by twenty-third day; not heard again (became ill)
	RW	40	.....	0.75–1.5	Crowed 17 times by fifty-eighth day; not heard again
	BB	.....	.....	1.0	Not observed to crow (became ill)
C	RR	28	.....	0.75	Crowed at least 6 times
	GG	34	.....	0.75	Crowed at least 4 times

of knowing how much crowing went on when no observer was present. Eight of the 12 injected birds are known to have crowed, while none of the more numerous controls was heard to crow. The crowing did not begin until there had been over 2 weeks of daily injections, and in 5 cases is not known to have continued until the end of the period of injections.

RY of flock A 5 was heard to crow on the last day of injection; **BY** of the same flock and **RG** of flock A 1, 2 crowed on the third day following the cessation of treatment. After this particular **BY** and this **RG** began crowing, each was heard almost daily until 3 days after cessation of treatment. Their crowing seemed to increase in quality as time went on, until a fine loud call was produced, which was, however, never so deep or prolonged as were those given by neighboring cocks.

The data offer a decided suggestion that crowing is related to dosage of TP. In this connection the case of **RW** of flock B 2 is suggestive. This bird received originally 0.75 mg. daily and was first heard to crow on the fortieth day of injection; it crowed again

2 days later and then, so far as we know, was silent for 6 days. At that time her dosage was doubled, and almost immediately she crowed six times in succession.

The crowing, like changes in the social order, did not begin until there had been 2 or more weeks of treatment. Both phenomena occurred during the same general period; they were not, however, invariably associated, since in flock B 2 both **GG** and **RW** crowed actively but did not rise in the peck-order of the flock. On the other hand, 1 hen in each of the A flocks gained 1 or more social reversals over flock mates without being heard to crow.

Courtship.—In flock A 5, **BY** was three times seen to give a somewhat abbreviated exhibition of courtship. She would drop one wing and flutter it rapidly against her side while sidling up to another hen, and at that same time would move one foot up and down in an exaggerated manner. This behavior was a rather good reproduction of the courting reaction of a breeding cock. **BY** was seen to react so on the thirty-eighth, forty-sixth, and forty-ninth days of injection to RW, RR, and RG, respectively. **BY** had recently moved into the α-position of her flock; RG and RW were tied for second place (both pecked 6 others; RG pecked RW); and RR ranked eighth. RW of flock B 2 gave a similar reaction to the poulard GY. This was seen on the thirty-sixth day of injection.

Egg-laying.—Trap nests were present in the pens at all times, and the birds gradually learned to use them for laying; however, the trap-nest records were never perfect. BR of flock A 1, 2 had been laying steadily with a trap-nest record that averaged 0.52 eggs per day for the 81 days that preceded the beginning of TP treatment on June 6. She laid an egg on June 7 and thereafter did not lay in a trap nest until after August 15. Her hormone injections had stopped on July 29. **RG** of the same flock laid 0.4 eggs per day in the same preinjection period, ceased laying when injection began, and started laying again in late August. Each of the control hens in this flock continued to lay throughout these summer months at about the same rate they had shown in the late spring. They averaged 0.26 trap-nest eggs per hen per day before the injections started and 0.39 eggs during each of the 54 days of injection while each was receiving 0.1 cc. of sesame oil daily. There were 0.06 eggs per hen per day which could not be definitely allocated to a given hen in the preinjection period, and 0.05 per hen per day during the injection periods.

Flock A 3, 4 was also injected during June and July. Of the hormone-treated hens, **GG,** which received 0.5 mg. of TP daily, laid its last egg on the second day of injection; **BG,** which received half that amount, laid on days 2, 32, 35, 37, and 44. The controls in this flock laid fewer eggs during June and July than they had in the spring months. With the exception of BR, which became broody, each of the controls laid an occasional egg throughout the injection period. They laid 0.18 unassigned eggs per hen per day before, and 0.17 during, injections.

Because of molting, none of the hens in flock A 5 were laying regularly during the period of hormone treatment (August 30–October 19). Each of the control hens, with a single exception, laid once or more during that period; and one, RG, laid at least 27 eggs. **BG** and **RY** of the hormone-treated hens had laid one or more eggs in late August. **BG** is not known to have laid again before her death on October 31. Neither **RY** nor **BY**, the other hormone-treated hens in this flock, are known to have laid again until in January. They both began laying regularly in February. Only three "unassigned" eggs were laid in this flock during the injection period.

The pullets of flock B 2 had not learned to use the trap nests at the time their treatment began; hence, data from this flock are inconclusive. So far as they go, the records

support our previous experience that injection with TP greatly retards or prevents egg-laying. One of the pullets laid in the trap nests once only during the period of treatment, although each had done so previously. Three of their 5 pen mates that lived through the test period continued throughout to use the trap nests with some regularity. **RW**, the surviving hormone-injected bird of this flock, began laying actively in February about 6 weeks after treatment was stopped.

THE CASE OF YY

In connection with a different set of experiments we ran one more test on the effect of TP, which was barely completed when this manuscript was sent to press; hence, the results could not be readily distributed among the proper categories in the preceding pages. This has advantages since the case in hand summarizes many of the points already covered and is, in many ways, a so-called "crucial" or "show" experiment, such as was more popular in an earlier period of experimental zoölogy.

TABLE 16

THE SOCIAL ORDER IN FLOCK B 1, 2 BEFORE AND AFTER TREATMENT

A (April 22)		B (June 26)		
Individual	Number Pecked	Individual	Number Pecked	Formerly Pecked†
BW	9	**YY**	8	0
RW	6	BW	7	8
BY	6	BR	4	5
GY*	5	RW	4	5
BR	5	BY	4	5
GG*	4	GG*	3	3
RG	4	GY*	3	5
RY	3	RG	2	3
BG*	2	BG*	1	2
YY	1			

* Poulards; GG is well masculinized. † RY died on June 13 and is not included.

Several deaths occurred among the highly inbred young Iowa birds of the B flocks during the winter. In early spring (March 24) the surviving members of flock B 2 were moved into the pen of the survivors of flock B 1. A month later they showed the peck-order which is listed in Table 16 (A). Beginning on April 22 **YY** was given 1.25 mg. of TP daily; BY and RY served as oil-injected controls. Injections were discontinued after July 9. It is of interest that RY, then called "RW" of flock B 2, had received 0.75 mg. daily from October 19 to December 4 and double that amount for the following 17 days and that she remained low in her social order.

YY was seen to begin revolting against her socially superior flock mates after 11 days of injection; 9 successful revolts were seen during May and early June. Her actual revolts began earlier, for after only 4 days of injections she revolted successfully and won a reversal over a sick hen, which later died and is not listed in Table 16. A week afterward she won her revolt against a sick poulard, which also is unlisted in the table. On May 7 **YY** defeated the poulards GY and BG, and on May 12 she won over BR. She

continued her upward climb in the flock order until on June 16 she ranked next to the top in number of subordinates, although she was still being pecked by her present despot (BW), by her former despot of flock B 2 (RG), and by the masculinized poulard GG. On June 17, **YY** rose over BW, on June 24, over GG, and became top bird of the flock on June 26 when she began pecking RG. YY has maintained this position to date (July 25).

Meantime the controls had been seen to give 2 unsuccessful revolts; and the masculinized poulard GG gave 2 reversals, 1 over a poulard (GY) and 1 over a sick bird. The effect of TP injections on the social position of **YY** is the more important, since, as shown on page 426, similar treatment when these particular birds were younger had not produced changes in the peck-order. At the beginning of the injections, **YY** was six months older than were the birds when the earlier treatment began; it was spring rather than autumn, with the difference in the egg-production cycle that this implies.

Other observed effects were all according to expectation and may be briefly summarized as follows:

1. The head furnishings became large and turgid, the face became very red, and the ear lobes became yellowish, as in cocks. The general carriage, especially of the tail, became more erect.

2. The size of the vent decreased almost to that typical of a cock, and the inter-pubic distance became less.

3. Spurs sprouted slightly.

4. No eggs were laid, however; **YY** was not laying just preceding treatment.

5. Crowing was first heard after 24 days of injections and continued steadily until 10 days after injection ceased and some 238 distinct crows had been recorded. During the last 7 days YY crowed only at dawn. Only one of our apparently normal hens has ever been seen to crow and this on only two occasions and in very rudimentary fashion. This individual laid an egg on one of the days she crowed. **YY** commenced calling hens to food like a cock on June 13 and was last noted to do this 3 days after stopping the injections.

6. **YY** began courting hens after 33 days of injection and a total of 86 such acts was recorded. Courtship was observed almost daily up to 7 days after the last injection, usually during initial encounters with strange hens. During several hundred such encounters only one apparently normal hen on only one occasion gave a reaction that could be said to resemble courtship.

7. **YY** won all 26 of her staged initial pair-contacts during the period of injection; all these were with normal hens. In previous series of such contacts she had lost except to poulards.

In the course of these staged encounters **YY** won one of the most severe combats we have yet seen between hens. She was introduced into a neutral pen with RY of flock D 1. RY is much heavier and older; has fairly high social status in her home flock; and is an experienced fighter, that usually wins. **YY** attacked, and the two fought vigorously for 21+7+2+2+12 seconds in successive onsets. Each time **YY** was beaten and retreated, but she kept forcing the fighting after short rests. Finally she retreated to the roost, as losers often do, and RY scratched litter on the floor below, a reaction which winners frequently give.

After a few minutes the observer opened the pen door, and at that **YY** jumped down to the floor near RY, which at once leaped up and perched on a side bar some distance

above, while **YY** scratched about below. Then the latter jumped up to the roosts and from there courted RY, which was approximately on her level and some 30 inches away. **YY** crowed successively at short intervals, then leaped to the floor and crowed again. RY reacted as though frightened and, when again placed on the floor, flew immediately to the roost. **YY** followed and courted RY, which pecked her on the head. A brief fight ensued, which terminated when RY fell off the roost. She leaped immediately to the side bar and made strong avoiding reactions when **YY** approached.

The latter was then removed from the pen for 5 minutes until RY recovered enough to begin feeding, whereupon **YY** was slipped under the partly opened door and RY at once flew up to the side bar. **YY** was again removed and later was again introduced, with similar results; and still later RY avoided **YY** when the two were alone together in a part of RY's home pen. To all appearances **YY** won by not knowing when she was beaten. Such persistent aggressiveness has been seen only once among other hens during the course of our observations.

DISCUSSION

In order to begin to understand the foundations of the social order in flocks of the common domestic fowl, one must have data concerning two radically different aspects, namely, those factors which initially determine the position of a given individual in the peck-order and those which make for the maintenance of the position, once it is established. These two sets of factors are not necessarily mutually exclusive, although they do present contrasting emphases. The initial position in the social order is largely determined by the relative aggressiveness of the individuals which compose the flock. Maintenance of position is associated with the factors which make for social inertia; such factors as memory and habit reinforce, and may entirely replace, the aggressive behavior patterns which are so important in the origin of a social order in hens.

In the present experimental analysis we have been investigating primarily the physiological basis of aggressiveness which determines which of two hens or pullets will dominate in a first contact with a stranger from another flock. Also, after the social order is established, we have been particularly interested in analyzing the factors which produce successful revolts that lead to a change in the established order. Although we had indications at the beginning and have since then accumulated more evidence that psychological factors are involved in aggressiveness, we have been directly concerned with a more obviously physiological approach.

The presence of male hormone is a potential source of aggressiveness. Cocks are more aggressive than hens or capons, and cocks dominate hens in a heterosexual flock. Hens apparently secrete male as well as female hormone; that is, they appear to be functional endocrine hermaphrodites, since the combs of ovariotomized fowl drop to a capon level (Domm, 1927). In normal hens both the medulla, which secretes androgenic material that affects the comb, and the cortex, which secretes estrogenic substance that affects the oviduct, respond to gonadotropic agents (Domm, 1937). Hence, the introduction of additional male hormone means increasing the amount of some material originally present rather than the introduction of a wholly foreign substance. Our choice of testosterone propionate (TP) for injection was determined by its chemical purity, its known physiological action on the nervous system as well as on other organ systems (Bize and Moricard, 1937), its relative efficiency (Breneman, 1939), and its availability. The results obtained have justified the selection.

In three flocks of adult hens the TP-injected individual moved from at or near the bottom to the top of the social system, and each of the hormone-treated hens showed one or more social reversals over normal, healthy flock mates. Such a change was shown by none of the controls which were similarly injected with sesame oil. In one flock of relatively young, highly inbred pullets the treated birds did not rise in their social order within the flock but they did win 75 per cent of their first contacts with strange, untreated normal pullets and lost only to 2 aggressive, experienced fighters—and probably then because of illness. Six months later, one of these inbred birds gave an almost diagrammatic response to treatment with TP. Finally, with young pullets in which the peck-order was just being established, the 2 birds receiving TP became dominant in their respective flocks.

Although it is now generally considered that comb growth in capons, when it occurs, is a result of androgenic stimulation, the presence of the appropriate androgen in suitable quantity is not the only factor that determines the size of a fowl's comb. Different breeds vary in this characteristic as in others; even our different flocks of white leghorns had more or less definite variations in comb size. Within limits the amount of sunlight to which an individual is exposed affects the size of head furnishings (Domm, 1930). With these facts in mind, it is not surprising that the observed correlation between size of comb and aggressiveness in first combats was no higher than we found it.

The increased comb growth which we obtained was to be expected from the work of others (Hamilton, 1938; Breneman, 1938; Emmens, 1938), as was also the evidence of a decrease in the rate of egg-laying (Schoeller and Gehrke, 1933) and the lack of maintenance of normal rate of growth in young birds (Breneman, 1939). The greater weight of the older TP-treated hens is in keeping with the experience of Bulliard and Ravina (1938) with ducks about 2 months of age and is opposed to the usual result with growing chicks, our own included.

Our findings concerning the stimulation of crowing differ from those of Hamilton (1938) with very young female chicks and afford a demonstration of the conclusion reached by Myers (1917), and confirmed by Appel (1929), that there is no apparent anatomical reason why the female leghorn fowl should not crow, provided it has the instinct to do so properly developed. Incidentally, these observations throw some light on the question of the ontogenetic origin of instinctive behavior.

Benoit (1929, p. 380) found, by using testicular grafts, that the threshold of hormone stimulation in the cock ran as follows, with the last mentioned having the highest threshold: "(1) Epididyme et canal déférent; (2) Organes érectile; (3) Inhibition du plumage; (4) Instinct sexuel; (5) Chant; (6) Ardeur combative." Benoit suggested that changes which involve the intervention of the nervous system have a higher threshold than do changes in plumage or in comb and wattle growth.

In our experiments with females, changes in comb size were most readily and certainly effected; a change in the rate of egg-laying typically came next; the initiation of crowing, next; and finally changes in social position. It should be noted that in the last, and for our purpose the most important, aspect of these studies we have not investigated the dosage that will cause a given hen to become more aggressive in staged initial contacts; our data regarding dosage apply only to changes in the well-established peck-order of hens that had been living together for some time.

These observations that changes in behavior have a higher threshold than do changes in structure run counter to the early conclusions of ecologists (Shelford, 1911) that with

animals it is easier to effect modifications in behavior than in anatomy. We have at present nothing to say about the relative ease of producing changes in behavior and structure in the so-called "lower" animals; obviously, the generalization can no longer be applied to the vertebrates.

There are many indications of the complexity of the factors which initiate and maintain the social order among flocks of hens. One of these indications is found in behavior following the cessation of injections. The sequence of events was as follows: the comb began decreasing in size, courtship vanished, crowing ceased,[7] and after a long interval egg-laying was resumed. In these respects the hens apparently returned to the *status quo ante*. However, the changes won in social position persisted as long as we followed the respective flocks—in the case of flock A 5, for at least 5 months.

Since social position in flocks of hens is not easily affected and changes come only after long treatment, this does not provide the physiologist with a satisfactory criterion for assay of hormone effectiveness. When the hormone begins to produce an increase in aggressiveness, the first revolts against social superiors are likely to fail even in cases when a few more injections will bring successful reversals. The first reversals shown by the low-ranking birds are likely to be the result of successes gained over those just above them in social rank and likewise relatively low in the social order. This is in part a result of the fact that birds near each other in social rank have more mutual contacts and in part because of the lesser aggressiveness one frequently finds in low-ranking hens. Only after a long period of injections, if at all, did our treated hens rise to the top of their respective flocks, and usually the last reversals were over the birds with the highest social status. It is entirely consistent with this picture that the social positions gained by the treated birds were maintained in the postinjection periods. The psychological factors which retarded her rise now helped maintain the hen in her newly acquired status.

It is fairly obvious, from what has been said, that the hen's relation to TP cannot be regarded as similar to that of chemicals in a test tube to which sufficient catalyst is added to cause the reaction to proceed to its logical conclusion. Among the other complicating factors are indications that recent experience in winning or losing encounters affects the outcome of the next pair-contact. These and other so-called "psychological factors" will be discussed in more detail in a later publication.

It is apparent that we have by no means solved the problem of the development and maintenance of social position even under the relatively simple conditions that exist in flocks of hens. We have shown that one physiological factor, the increase in the amount of male sex hormone present—at least to the extent that natural male hormone acts as does TP—if continued for some weeks, increases the likelihood that individuals so treated will better their position in the social order of the flock.

Our experiments were directed toward this one end, and we have not undertaken the arduous task of investigating the physiological mechanism(s) which finally produce changes. For that matter, we do not know with certainty the effect produced on an experienced hen by the physiognomy of a strange bird which possesses the high comb and general appearance of other birds which have recently held the peck-right over her. As an example, the behavior of BR_2 in contact with the large-combed but nonaggressive GG_1, as outlined in Table 13, suggests that this possible factor is not negligible.

[7] Domm (1935) reports that the precocious crowing of male chicks stopped within 48 hours after the cessation of daily injections of hebin.

Finally, we have been considering some aspects of the social order in flocks of pullets and hens primarily because of our interest in the behavior and social life of birds. We have the distinct impression, however, that in working with this problem we are making an attack on some of the fundamental aspects of group living and graded social position as found in many vertebrates.

SUMMARY

1. Testosterone propionate (TP), injected into low-ranking individuals in these flocks of white leghorn hens, produced a rise in social status in each adult that was treated; and an injected individual eventually occupied the top position in each flock.

2. With one flock of younger pullets, in which the peck-order had just become fixed after an extended period of fluctuations, similar injections produced no changes in the social position within the flock. However, the hormone-treated pullets were more aggressive and successful in initial contacts with strange birds than they were before the injections.

3. In two flocks of battery-reared young pullets studied while the social order was beginning to be established, the injected birds came to dominate each flock.

4. In initial pair-contacts with strange birds, the hen or pullet with the larger comb had decidedly the better chance of dominating the situation; comb size is generally considered to be an index of the amount of male hormone present.

5. Other observed effects of TP include increased size of comb, retardation or suppression of egg-laying, initiation of crowing, and, with three hens, of courtship. These changes vanished soon after the cessation of treatment. Higher social position, once won, was retained.

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Business correspondence should be addressed to The University of Chicago Press, Chicago, Ill.

Communications for the editors and manuscripts should be addressed to W. C. Allee, Managing Editor of PHYSIOLOGICAL ZOÖLOGY, The University of Chicago, Chicago, Ill.

The articles in this journal are indexed in the *International Index to Periodicals*, New York, N.Y.

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